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SCIENTIFIC PROCEEDINGS

THIRTY-SEVENTH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW YORK CITY, DECEMBER 26, 27 AND 28, 1935

Headquarters: Hotel Pennsylvania

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ABSTRACTS*

Report of the Committee on Bacteriological Technic: Progress During 1935. H. J. Conn, *Chairman;* Victor Burke, Barnett Cohen, M. W. Jennison and J. A. Kennedy, *Members.*

At the 1934 meeting of the Society permission was obtained to print and put on sale a new Descriptive Chart, drawn up by a sub-committee consisting of M. W. Jennison and the Chairman of the general committee. Various suggestions for improving this new edition, however, were made at the meeting and as the result it was several months before the final draft was ready for printing. The new chart was finally printed in July and about 11,000 copies have been sold up to the date of this report (November 11, 1935).

During 1935 Leaflets VI, VII and VIII of the Manual of Methods for Pure Culture Study of Bacteria have been revised. These deal, respectively, with: biochemical methods, the study of pathogens, and serological methods. A new index to the entire Manual has also been issued during 1935. These revised leaflets and the index have all been issued in the quarterly journal "Pure Culture Study of Bacteria" which is published by the Committee as a continuation service of the Manual.

From a business standpoint the Committee still has a favorable report to make. The total sum turned in to the treasury as profits for 1934 was $390.45, the largest for any year except 1927 and 1930, which were $426.00 and $457.00, respectively. The profits turned in for the first 10 months of 1935 have been $355.00 which is very near to the $345.00 reported for these same 10 months in the latest annual report.

GENERAL BACTERIOLOGY

G1. Centrifugal Fractionation of Heat Resistance in a Spore Crop. J. Yesair and E. J. Cameron, Research Laboratories, National Canners Association, Washington, D. C.

A method has been devised by which crops of different types of

* This number of the Journal has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, due to restrictions of time imposed by the printing schedule.

heat resistant bacterial spores may be fractionated into various crop fractions of varying resistance to heat.

A "Foerst" supercentrifuge was operated at a bowl speed of 43000 r.p.m. Fractionation was accomplished by centrifuging the spore-containing medium a number of times and collecting the individual spore residues from the centrifuge cup. The rate of feeding was also varied.

The following is a typical example which illustrates the fractionation method and its result. A pork broth medium in which spores of a putrefactive anaerobe had been produced was centrifuged at a rate of one liter in 15 minutes. The effluent was recentrifuged at a rate of one liter in 30 minutes. This effluent was again recentrifuged at a rate of one liter in 60 minutes. Thus three spore fractions were obtained. Spores from the first lot suspended in phosphate of pH 7.0 survived 20 minutes heating at 115°C. and were destroyed in 25 minutes. The second lot of spores, when heated in the same concentration and under the same conditions, had a survival time of 10 minutes at 115°C. and was destroyed in 15 minutes. The third lot survived 5 minutes and was destroyed in 10 minutes. Thus a wide spread in resistance was obtained among spores of a single spore crop.

Experiments yielding qualitatively similar results have been conducted with a thermophilic anaerobe and a thermophilic "flat sour" organism. The results suggest that resistance variations may be correlated with the specific gravity of individual spores, but this has not been determined with certainty. There is the alternate possibility that the less resistant spores are larger and are thus subject to greater resistance by the suspending medium. Finally, it has been suggested that the presence of spore clumps may be a factor, although this is regarded as unlikely in view of the technique which was used in the preparation of suspensions.

G2. The Ultra-Violet Radiation of Bacterial Spores. HAROLD R. CURRAN, Bureau of Dairy Industry, Washington, D. C.

Spores were exposed in aqueous solution to light generated by a quartz mercury-vapor lamp.

The destructive action of ultra-violet light upon spores may be enhanced by the use of metallic reflectors. The comparative usefulness of different metals for this purpose was determined.

Some of the reaction products of irradiation were studied and the possible relationship of these to the lethal action of ultra-violet light

was considered. Under favorable conditions hydrogen peroxide is a powerful sporocide and extremely small concentrations of this substance will prevent the germination of many species. The effectiveness of hydrogen peroxide either as a bactericidal or bacteriostatic agent is closely correlated with the presence or absence of active catalase or peroxidase.

Other factors which may to some extent influence the sporocidal activity of ultra-violet light include: the treatment of the irradiated material subsequent to exposure, concentration of oxygen in the exposed material, presence or absence of air during exposure, and the temperature of the irradiated material.

G3. Dissimilation of Glucose by the Lactic Acid Bacteria. M. E. NELSON AND C. H. WERKMAN, Department of Bacteriology, Iowa State College, Ames.

Dissimilation of glucose by typical heterofermentative lactic acid bacteria (*Lactobacillus lycopersici*, *Lactobacillus acidophil-aerogenes*) yielded CO_2 equivalent to the sum of acetic acid and ethyl alcohol. Lactic acid was secondarily fermented to acetic acid and CO_2. The latter reaction requires formation of a reduced product—glycerol was found equivalent to twice the acetic acid, in agreement with the theory involving liberation of active hydrogen by dehydrogenation of lactic to acetic acid and CO_2.

Pyruvic acid has been identified as an intermediary in the fermentation of lactic acid. Fermentation of pyruvic acid yields acetic, carbonic and lactic acids in equimolar quantities (dismutation).

Reducing properties of heterolactics are pronounced: (1) levulose to mannitol, (2) acetaldehyde to ethyl alcohol. The latter reduction was accompanied by an increase of acetic acid and CO_2 equivalent to one-half of the acetaldehyde reduced, and a decrease of lactic acid formed from glucose, equivalent to the increase in acetic acid. (3) Acetylmethylcarbinol was reduced to 2,3-butylene glycol by heterolactics (*Lactobacillus lycopersici*, *Leuconostoc dextranicus*). The homofermentative lactic, *Lactobacillus plantarum*, did not bring about the above reductions. A scheme of dissimilation of glucose is proposed based on the relationships established by the data involving a splitting of the 3-C intermediary into lactic acid and into acetaldehyde, CO_2 and 2H. Secondary fermentation of the lactic acid yields acetic acid, CO_2 and 2H. Acetaldehyde is reduced to ethyl alcohol. Glycerol is formed probably by hydrogenation of a 3-C intermediary.

G4. Phosphoglyceric Acid in the Dissimilation of Glucose by Citrobacter freundii. C. H. WERKMAN, E. A. ZOELLNER, HENRY GILMAN AND H. REYNOLDS, Departments of Bacteriology and of Chemistry, Iowa State College, Ames.

Phosphoglyceric acid has been isolated as an intermediary in the dissimilation of glucose by *Citrobacter freundii* using the technic of Neuberg and Kobel adapted to bacterial requirements. The purified acid has been identified by its specific rotation($[A]_D = -13.33°$), carbon and hydrogen analyses, and determination of the barium/phosphorus ratio.

Additional evidence for the occurrence of phosphoglyceric acid as a normal intermediary in bacterial dissimilation of carbohydrates was found in its conversion by *Citrobacter freundii* to pyruvic acid. The organisms were grown in peptone-glucose medium at 37°C. for 20 hours, centrifuged and washed three times, and then used in 1.2 per cent concentration, moist weight. Authentic phosphoglyceric acid (0.8 per cent) prepared from glucose by action of yeast, and one per cent toluene constituted the medium. Control flasks contained no added phosphoglyceric acid and at no time did they show the presence of pyruvic acid. Flasks with phosphoglyceric acid were negative at 1 hour but at 3 hours pyruvic acid was definitely present, increasing in concentration until at 12 hours the fermentation was stopped. The pyruvic acid was precipitated with 2,4-dinitrophenylhydrazine. The purified yellow precipitate had a melting point and mixed melting point with an authentic derivative of pyruvic acid of 213°C., uncorrected.

These results suggest that the mechanism of muscle glycolysis of Embden and of Meyerhof may have its counterpart among bacteria.

G5. Aerobic Dissimilation of Lactic Acid by the Propionic Acid Bacteria. H. G. WOOD, CARL ERB AND C. H. WERKMAN, Department of Bacteriology, Iowa State College, Ames.

A constant pressure type macro-respirometer using 500 ml. of medium has been developed for the study of the aerobic dissimilation of microörganisms. Quantitative analyses of products are carried out by macro methods. CO_2 is determined by absorption in KOH. Oxygen uptake is measured by volume change in a calibrated burette. The apparatus is of special value in the study of aerobic metabolism. Carbon and oxidation-reduction balances of aerobic dissimilations can be calculated, assuming the oxygen utilized is hydrogenated to water.

The aerobic dissimilation of lactic acid has been studied using non-

proliferating *Propionibacterium arabinosum*. The lactic acid was buffered with phosphate at pH 6.0. Temperature was 30°C. and time of fermentation from 30 to 70 hours. Active utilization of oxygen occurred, the lactic acid being converted to pyruvic, acetic, propionic and carbonic acids. The carbon of the lactic acid fermented was completely accounted for in products. The oxidation-reduction balances were satisfactory assuming hydrogenation of oxygen to water. Oxygen may function as a hydrogen acceptor in the propionic dissimilation. In certain experiments the pyruvic acid accounted for 70 per cent of the substrate carbon. Additional evidence is thus furnished of the intermediary behavior of pyruvic acid in the propionic acid dissimilation.

G6. Effectiveness of Hot Hypochlorites of Low Alkalinity in Destroying Mycobacterium tuberculosis. S. M. COSTIGAN, J. W. YATES, W. A. HADFIELD AND E. C. MCCULLOCH, General Laboratories, Inc., Philadelphia, Pa.

The growths from two month old cultures of human strains of *Mycobacterium tuberculosis* were washed from solid media with normal saline and the suspension was standardized so that the addition of 2 cc. of the organism suspension to 20 cc. of the disinfectant dilution would yield 20,000,000 organisms in each cubic centimeter withdrawn for inoculation into guinea pigs. Each dilution of hypochlorite was maintained at the designated temperature for a short period before the organism suspension was added. At intervals of one-half, one, two and one-half and five minutes, 2 cc. were withdrawn and divided between two centrifuge tubes each containing 5 cc. of N/10 sodium thiosulphate to neutralize any remaining hypochlorite. After centrifuging for 10 minutes, the liquid was decanted and the cubic centimeter remaining in the bottom of each tube inoculated subcutaneously into the left inguinal region of a guinea pig. At the same time, parallel controls were made in which the hypochlorite was omitted. The guinea pigs were autopsied at the end of 10 weeks and acid-fast stains made of the left inguinal lymph glands and other organs showing lesions.

The data indicate that hypochlorites in a concentration to yield 50 parts of available chlorine per million, kill *Mycobacterium tuberculosis* in two and one-half but not in one minute when exposed at 50°; in 60 but not in 30 seconds at 55°; and in 30 seconds or less at 60°C.

Solutions containing 200 parts of available chlorine per million kill in 60 but not in 30 seconds at 50°; and in 30 seconds or less at 55° and 60°C.

G7. The Similarity between the Specificities of Disinfectants against a Filtrable Virus and Gram Negative Organisms. ERNEST C. MCCULLOCH, Pennsylvania Salt Manufacturing Co., Philadelphia.

A close parallel was noted between the relative efficiencies of phenol, liquor cresolis, formaldehyde, sodium hypochlorite, sodium hydroxide and several dyes against the virus of fowl pox contained in tissue debris and against Gram negative organisms grown in a medium containing fecal debris.

The delayed reaction of formaldehyde-treated virus has an exact duplicate in the delayed growth of formaldehyde-treated *Eberthella typhi* and *Salmonella pullorum.*

The coefficients of temperature and dilution of each disinfectant were approximately the same when tested against the filtrable virus and the Gram negative organisms. The experimental data suggest that previous reports of the great resistance of filtrable viruses to chemical disinfectants may be due to the protective action of the organic matter in which they are contained.

G8. Evaluation of Mercurial Antiseptics in the Presence of Serum. DOROTHEA E. SMITH, E. J. CZARNETZKY AND STUART MUDD, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia.

Several mercurial antiseptics have been compared for their germicidal action in saline and in 80 per cent serum. An accurate method of plating has been used, and the time and concentration required for 50 per cent killing of a culture of smooth *Bacterium typhosum* has been taken as a basis of comparison.

Metaphen, Merthiolate, Mercurochrome and bichloride of mercury all combine with serum proteins. When maximum combination is secured the resulting compounds possess no antiseptic action. The mercury content found by analysis of the equilibrium germicide-protein compound is the same, within the limits of experimental error, for all four germicides. Equilibrium has been approached in both directions. In one case an excess of mercurial was used, and in the other an excess of serum proteins. In the latter case, the rate of combination was determined by adding the calculated amount of mercurial and noting the time when the mixture no longer possessed antiseptic action. The ratio of the rate with which the mercury of the mercurials combines with bacteria and with serum proteins is more favorable in the case

of some of the mercurials than with the others. These compounds kill bacteria, then, only in proportion to their mercury content. The decrease in germicidal activity when used in serum is equal to the amount of mercury chemically combined with serum. When used in saline, the killing power of the antiseptics is directly proportional to their concentration, but when used in serum, the killing power is proportional only to the concentration of antiseptics not combined with the serum proteins. Approximately 0.4 grams as mercury of any of these mercurials can be bound by one liter of normal horse serum.

It follows that the effect of serum is such that neither Metaphen, Merthiolate, Mercurochrome, nor bichloride of mercury may be used as an intravenous antiseptic.

G9. Studies on the Bactericidal Action of Mixtures of Phenol and Merthiolate. CAROLYN ROSENSTEIN FALK AND SOPHRONIA APLINGTON, Bureau of Laboratories, New York City Health Department.

In a previous paper the mixture of 1/20,000 Merthiolate and 1/400 phenol proved to be an efficient bactericidal agent for organisms commonly found as contaminants in biological products. At this time we are reporting on studies made to determine the exact time required to destroy these organisms, the minimum amounts of each preservative used in the mixture required to destroy these organisms, and the optimum temperature at which these reactions take place.

The bactericidal action of Merthiolate 0–1/10,000 and of phenol 0–1/200, alone, and in mixtures of different concentrations, on *Bacillus pyocyaneus*, staphylococci, and diphtheroids, isolated from contaminated biological products, has been studied at time intervals varying from 2 minutes to 7 days at 37°C., 20°C., and 5°C., in normal horse serum and in 0.85 per cent saline.

Selective action of the individual preservatives tested on the organisms studied was again observed and details of the killing time are given. The bactericidal action was most rapid at 37°C. (the highest temperature tested) and slowest at 5°C. In the presence of serum, to obtain the intensified action of the mixture of the preservatives, phenol must be used in a dilution of not less than 1/400 and Merthiolate in a dilution of not less than 1/40,000. If used in higher dilutions, the bactericidal power of the mixture is no stronger than that of the strongest preservative in the mixture used alone. Results are much less constant when the reaction takes place in the presence of saline. Bac-

tericidal action is however apparently completed in 24–48 hours on all the organisms tested, when the mixture of 1/800 phenol and 1/80,000 Merthiolate is used, at all the temperatures studied in these experiments.

G10. A Practical Evaluation of Antiseptics for Use in Oral Hygiene. M. A. JACOBSON, Chicago, Illinois.

The present F. D. A. Method for testing antiseptics to be used in oral hygiene has been criticized for the following: (*a*) failure to place sufficient importance on the strength or dilution of the product; (*b*) failure to stress the importance of the duration of application; (*c*) the F. D. A. Method does not approximate the practical conditions of use of antiseptics in oral hygiene.

The author recommends that the regular F. D. A. Method (Circular No. 198, published by the United States Department of Agriculture in December, 1931) be modified to the extent of simulating in a practical way the conditions of use of a product for use in oral hygiene. The proposed test includes a 20 per cent sterile saliva for each dilution tested. Also, the proposed test introduces a new practical terminology in the evaluation of antiseptic potency, namely, the "*Antiseptic Unit.*" "In oral hygiene a product is said to possess one antiseptic unit when four cubic centimeters plus one cubic centimeter of sterile saliva kills one-half cubic centimeter of a 24-hour broth culture of the standard F. D. A. *Staphylococcus aureus* in less than one-minute exposure."

Depending upon product dilutions of 1:2, 1:3, 1:4, 1:5, et cetera, (each 5 cubic centimeters containing 1 cubic centimeter of sterile saliva) killing 0.5 cubic centimeter of a 24-hour broth culture of *Staphylococcus aureus* in less than one minute exposure are claimed to possess either 2, 3, 4 or 5 antiseptic units, respectively.

The antiseptic unit evaluation of antiseptics for use in oral hygiene proves to have many advantages over the present F. D. A. Method.

G11. An Improved Method for Determining the Antiseptic and Bactericidal Powers of Gases and Vapors. J. L. POSNER, A. R. FODOR AND JOHN T. MYERS, Bendiner & Schlesinger, Inc., N. Y. C. Research Department.

The following method seems simpler and more accurate than any now employed:

Put the reaction mixture in a 50-cc. Erlenmeyer flask, stopper, and allow gaseous equilibrium to develop. Pass a bead headed pin through

a cork so it will project about 1.5 inches into the flask. Refold a sheet of lens paper in halves several times; place rough filter paper on top and bottom; and drive a cork borer through with a blow, cutting disks about 11 mm. in diameter. Autoclave the disks in a petri dish and transfer one at a time to a petri dish containing a broth culture of a test organism, as staphylococcus. After soaking 30 minutes place them with sterile tweezers between about 10 layers of sterile filter paper. When dry, suspend them, centrally pierced, on the pin, using a number twice that of the desired numbers of transfers, and insert into the prepared flask.

At timed intervals remove cork and disks; insert another stopper; drop one disk into a tube of broth and place another on an agar plate, then remove it; reinsert the cork and disks, for another time exposure.

After 24 to 48 hours incubation at 37°C., the results in the broth cultures give the end point of germicidal action and the plates with decreasing numbers of discrete colonies indicate the rate of destruction.

Work done so far is based on mixture of trioxymethylene and $NaHSO_4$. It should be possible to compare unknown gases with a standard as in phenol coefficient determinations.

G12. Self-Stimulation of Mold Cultures by Reflection of Mitogenetic Rays.
JEAN FERGUSON, Cornell University.

When mold spores on agar in a petri dish are kept under a cover of glass or quartz, they germinate more rapidly than with a cover of black paper, or of sterile agar, or without cover. The increase in germinated spores is 20 to 80 times the probable error. The stimulation can be explained by the observation of Wolff and Ras that reflected ultraviolet light or reflected mitogenetic rays produce a stronger mitogenetic effect than the direct rays; this is probably caused by polarization through reflection. It seems therefore, that the first germinating mold spores radiate, and that the reflection of these rays from the cover of the petri dish stimulates other spores. Neither black paper nor agar reflect light, and hence there is no stimulation.

The failure of many investigators to obtain mitogenetic effects may be explained by this self-stimulation. A culture in a glass-covered petri dish is exposed to the reflection of its own radiation which might be as strong as that of the supposed sender. The agreement between control and experiment would not prove the absence of radiation in the sender because both the control and the test plate might have been radiated.

G13. Physical Factors Influencing Bacterial Growth. C. S. MUDGE AND F. R. SMITH, College of Agriculture, University of California, Davis.

In the study of bacterial growth (population growth) the emphasis heretofore has been placed more on the nutritional aspects of the environment than on the physical. Among the important physical factors influencing bacterial growth are pH, E_h, surface tension, viscosity and buffer intensity. Buffer intensity has been studied in this work. Using the Van Slyke buffer index "β," or db/dpH, the titration curve for the various buffers was first determined and from these data β was calculated. This was done for the various peptones on the market as well as for the organic and inorganic buffers (citrates, casein and phosphates). Differences were found in certain of the buffers in that some went through maximum values for the buffer index and others through minimum values when the pH varied between 7.0 and 4.5. Media could be adjusted to any buffer intensity by use of a graph in which β is plotted against concentrations for all of the buffers studied. The fact that milk has a β of 0.02, while most common culture media have a β of 0.001 seemed significant. When media made with a β approximating that of milk were inoculated with *Streptococcus lactis*, better growth resulted than that in the unadjusted media.

The bacteriological phase of this work has not been completed.

G14. The Relationship between the Plate Count and the Direct Microscopic Count of Escherichia coli during the Logarithmic Growth Period at Various Temperatures. M. W. JENNISON, Department of Biology and Public Health, Massachusettes Institute of Technology.

In broth cultures at optimum temperature, even during the period of maximum rate of reproduction, the numbers of bacteria as determined by plate count generally are less than are enumerated by direct microscopic examination. On the basis of such observations, it is commonly stated that aside from clumping, a certain percentage of the organisms is dying out *in the culture.* Actually, such data merely indicate that some of the bacteria, after removal from the culture, do not develop into colonies upon plating out on agar. The object of this preliminary study was to investigate the relationships between counts, with a view to elucidating more clearly the reasons for the discrepancies.

Series of culture tubes containing 9 ml. of broth were inoculated with the same number of cells per ml. from a broth culture of *Escherichia*

coli. The tubes were incubated at 22°, 27°, 32° and 37°C. At exactly 2-hour intervals two tubes were removed at each temperature, shaken, and the contents pooled before sampling. A portion was immediately formalized for the direct count, and another portion immediately plated. The temperature of the dilution water (distilled) was not over 22°C. Plates were incubated at 37°C. for 48 hours before counting. The light-field, direct microscopic count was done in a Helber counting chamber, after staining the organisms with methylene blue. A *total* count was made of the total number of individual cells, and a *clump* count of single cells and groups of cells. The reliability of the plate and of the microscopic count was estimated to be approximately the same, the average deviation from the mean counts being about 5 per cent of the mean. Because of the fact that during the early part of the logarithmic growth period the density of cells was too low to make accurate direct counts, data for comparison include the latter part of this period and one subsequent 2-hour growth interval.

The relationship between counts made two hours after cessation of the maximum rate was essentially the same as during the maximal period. The *plate* count varied from 56 to 80 per cent of the *total* count at various times and temperatures, while the *clump* count varied from 58 to 90 per cent of the *total*. Such data are to be expected with any organism which forms clumps. Comparing the *plate* count with the *clump* count, it was found that at 37°C., with three sampling periods, the plate count averaged 83 per cent of the clump; at 32°C., with three sampling periods, 88 per cent of the clump; at 27°C., with four sampling periods, 84 per cent of the clump; and at 22°C., with five sampling periods, 100 per cent of the clump count. At the higher temperatures some of the bacteria present in the culture do not develop into colonies on agar plates, wheras at 22°C. all are viable, *distilled* water being the diluent in all cases. With *phosphate* broth as the diluent for plating, the plate count was close to 100 per cent of the clump count at both 22° and 37°C. During the logarithmic phase at optimum temperature *Escherichia coli* does not die out appreciably *in culture*, but some cells may die in distilled water during plating.

G15. The Relationship of Certain Respiratory Enzymes to the Maximum Growth Temperatures of Bacteria. O. F. Edwards and L. F. Rettger, Department of Bacteriology, Yale University.

The maximum temperatures permitting macroscopic growth were determined for 106 strains of the genus *Bacillus* in beef extract broth,

in one per cent dextrose beef extract agar shake tubes, and on beef extract agar slants. In 1000 trials no significant difference in the weighted average maximum growth temperature value on the 3 media was demonstrated. Morphological, physiological and cultural characteristics differentiated the strains into 18 groups.

Washed suspensions of each strain grown on beef extract agar were divided into 9 equal portions and heated for 24 hours through a temperature range of 35° to 75°C. at 5-degree intervals. The heated suspensions were then tested for hydrogen peroxide and for the presence of the different enzymes.

A thermo-stable peroxidase was found in each strain, using as reagents (in the presence of fresh aqueous extract of horse-radish) one per cent guaiac in 95 per cent alcohol, 0.5 per cent benzidine, 0.5 per cent o-tolidine in 50 per cent alcohol, and an aqueous solution of 2-7 diamino-fluorene-HCl prepared in our laboratory according to the method of Schmidt and Hinderer. No evidence of hydrogen peroxide formation could be detected, although in the presence of a M/10,000 solution of known hydrogen peroxide (control) a strong positive test was given.

An acceleration of the oxidation of one per cent solution of paraphenylene-diamine-HCl in KH_2PO_4-NaOH buffer, pH 7.3, served as a positive test for indophenol-oxidase, providing boiled cell suspensions and cells to which M/50 KCN had been added were negative. Evolution of gas following the addition of hydrogen peroxide to a suspension of the cells at pH 7.3 indicated the presence of catalase. The presence of succino-dehydrogenase was detected by adding 0.2 cc. of 1/50,000 sterile methylene blue to a mixture of 0.5 cc. of cell suspension, 0.5 cc. of sterile KH_2PO_4-NaOH buffer, pH 7.3, and 0.2 cc. of sterile 5 per cent neutralized succinic acid. The tubes were placed in a Novy jar, evacuated and incubated at 37°C. Decolorization indicated the presence of this enzyme.

The minimum temperatures of destruction of these 3 thermolabile enzymes were determined for each strain. Graphs were constructed by plotting the weighted average maximum temperatures of growth against the weighted average minimum temperatures of destruction for each enzyme for each of the 18 groups. The plotted data indicated a linear relationship for each enzyme. The coefficients of correlation for each enzyme were computed. The following values were obtained: for indophenol-oxidase, 0.8418; for catalase, 0.7222; and for succino-dehydrogenase, 0.4722. The first two indicated a close relationship

between the minimum temperatures of destruction of indophenol-oxidase and catalase and the maximum temperatures of growth. A less close relationship was shown between the minimum temperatures of destruction of succino-dehydrogenase and the maximum temperatures of growth. The combined effect of the minimum temperatures of destruction of all 3 enzymes upon the maximum temperatures of growth is measured by the coefficient of multiple correlation. A value of 0.8466 was obtained, which must be regarded as a close relationship.

An extension of these methods to other non-pathogenic microörganisms, including Gram-negative rod forms and Gram negative and Gram-positive cocci, with the purpose of obtaining further insight into the mechanism of bacterial growth at higher temperatures, is now in progress.

G16. Oxidation-Reduction Potentials of Certain Anaerobic and Facultative Anaerobic Bacteria: I. Eh:pH Relationship and a Possible Explanation for the Phenomenon of Double Reversion of Potential During the Apparent Logarithmic Phase. II. Preliminary Observations on the Differentiation of Lactobacilli of Intestinal and Bucal Origin. R. W. H. Gillespie and L. F. Rettger, Department of Bacteriology, Yale University.

During studies on O/R potentials in cultures of various anaerobes and facultative anaerobes, we have, as previously reported, accumulated a considerable amount of evidence to indicate that different species of the *Clostridium* genus establish significantly different levels of final reduction potential. The present report deals with observations which illustrate the importance of the relationship between H-ion concentration (pH determinations, accurate to within 0.02 to 0.03 unit, were made by means of the glass electrode) and O/R potential in analytical work. The influence of changes in pH on electrode potentials seems to have been neglected by many investigators. Observations which suggest a method for distinguishing different types of lactobacilli are also presented.

I. Cultures of lactobacilli in unbuffered tomato broth medium were found to illustrate the Eh:pH relation quite clearly because of the pronounced changes in pH which occur during the growth of these organisms in this medium.

A lactobacillus strain isolated from the mouth and characterized by marked activity with respect to growth and acid production exhibited at first a period of increasing reduction intensity. However, before the

final maximum reducing level was reached a sharp reversion of direction of drift occurred. Shortly thereafter the negative drift was resumed. The reversion of potential took place during the period of most rapid change in pH. This phenomenon has been observed on several occasions in cultures of various bacteria, especially *Cl. botulinum*. The reversion of potential has been reported by several others, but as far as we are aware no successful attempt has been made to attribute this reversion to the direct effect of pH change. Hewitt believes that the two phenomena are not directly related. We have obtained, in at least two instances, evidence strongly favoring change in H-ion concentration as the direct cause. The glass electrode is being adapted for use in the oxidation-reduction electrode vessel to facilitate the study of the Eh:pH relationship in bacterial cultures.

Under practically identical cultural conditions, change in O/R potential and H-ion concentration of cultures of two strains of *Lactobacillus acidophilus* were followed. No double reversion of potential occurred, probably due to the fact that acid production was more retarded than in the mouth strain culture, not beginning until after the maximum reduction potential was reached. However, as acid accumulated in the cultures a positive drift occurred. The drift was proportional to the magnitude of pH change, and the changes took place simultaneously. The observations on the Eh:pH relationship in cultures of lactobacilli are supported by results of a similar nature with *Escherichia coli* and *Salmonella pullorum*. Under the conditions of the experiment the mouth strain established an appreciably lower final pH than did the two intestinal strains.

II. In a buffered medium of low carbohydrate content the development of the lactobacilli caused little change in pH. This factor being eliminated, the strains of oral and intestinal origin were differentiated by a rather wide margin of final reduction intensity, a margin which, if further substantiated, should make their differentiation by means of the dye indicators practical.

G17. Metabolic Activity and Cell Volume of Salmonella gallinarum at Various Phases of the Culture Cycle. EVELYN HUNTINGTON, Yale School of Medicine.

Mooney and Winslow have suggested that the high rates of metabolic activity per cell noted in the lag and logarithmic growth phases result partly but not wholly from an increase in cell size. In order to throw light upon this problem samples of *Salmonella gallinarum* cultures (of

which the growth and CO_2 production were determined hourly) were used. Smears made from these samples were stained with dilute Ziehl's fuchsin and cell sizes determined by using a filar micrometer.

Cell size reached a maximum at the end of the lag phase when mean cell volume was 3 to 4 times that of the original cells. The decrease in volume following the maximum was more gradual than the increase, with mean cell volume at the end of the logarithmic growth period one-third to one-fourth greater than the initial value.

Rates of CO_2 production per unit volume of bacterial protoplasm exhibit an increase in the lag and early logarithmic phases over the initial and terminal rates as do the rates calculated per cell; but this increase is substantially less than that reported for the rate of CO_2 production per cell. For instance, in 1 per cent peptone the maximum hourly rate per unit bacterial substance was 15 times larger than the initial rate, while the maximum rate of CO_2 production per cell was 40 times its initial rate. This same general relationship is demonstrable in a medium of 1 per cent peptone with 0.5 per cent lactose (a sugar not utilized by this organism). Thus further support is given to the conception of a period of physiological youth during which bacterial cells exhibit an increase not only in size but also in metabolic activity.

G18. Optical Density as a Criterion of Growth in Suspensions of Bacteria.
HAROLD MESTRE, Yale School of Medicine.

Increase in volume, wet or dry weight, or in the number of cells, total or viable, per unit volume of suspension have commonly been used as criteria of growth in cultures of microörganisms in liquid media, Various indices of metabolism have also been employed. In general, each of these criteria gives a different picture of the growth cycle, and this picture is further varied by the particular technique used. With suitable organisms, the optical density, which is the common logarithm of the opacity (or of the reciprocal of the transmission) of a suspension, is mainly a function of the total surface area of cells per unit volume, when determined with a photometer of extremely small angular aperture. Depending on the instrument used, the optical density is also, to some extent, a function of the shape, relative index of refraction, and other cytoplasmic characteristics of the cells.

Changes in the optical density of a growing suspension may be followed practically continuously, and the data can be plotted within a few minutes of the observation. The precision is far greater than with the more usual methods, and the procedure is wholly objective.

With adequate control of variables, the optical density curves give a highly reproducible picture of growth, and their form is a characteristic function of the specific organism, the physiological state of the inoculum, the medium, the temperature, and the degree of aeration. The effects of these variables will be illustrated from data secured in collaboration with Dr. H. H. Walker, and a partial interpretation given of the optical growth curves for *Escherichia coli* in terms of some of the other criteria of growth.

G19. Numbers, Size and Optical Density at Various Phases of the Bacterial Culture Cycle. H. H. WALKER, Yale School of Medicine.

The strain of *Salmonella gallinarum* employed by Huntington and by Mooney and Winslow, et al., was grown in aerated media (1 per cent peptone and 1 per cent peptone plus 0.5 per cent lactose) at 37°C. Growth in numbers was determined at hour or two hour intervals, simultaneously, by standard plating methods and by darkfield chamber direct counts of discernible individual cells and cell-aggregates (pairs, chains or clumps). Growth in cell size was determined by the stained-smear filar-micrometer method by Miss Huntington. Growth in optical density was determined with the precision photometer by Dr. Harold Mestre. All the above measures of growth were applied concurrently to portions withdrawn at each sampling interval from the same growing cultures. Six duplicate cultivations were studied for each medium and the mean results presented.

Semi-logarithmic curves plotted for the product of direct counts and mean surface areas (or volumes) and for optical density revealed a general parallelism and similar slope extending well through the hours of active multiplication, but excepting the very early and late hours of each day's run, where some divergence appeared. In general, direct counts paralleled plate counts during active growth but their absolute values were approximately twice as high. In lactose media, plate counts fell much more sharply below direct counts in the early logarithmic phase. This may account for certain abnormally high CO_2-per-cell rates obtained by Mooney and Winslow, and indicates that even the CO_2-per-cell-volume rates of Huntington may be subject to revision downward at those points. After 24 hours, direct counts vastly exceeded plate counts but here the plate counts are probably more significant than the direct counts in relation to metabolic activity. However, relationship between CO_2 output and unit volume or surface, even with the several further allowances for size permitted by this

study, suggests that the high output per cell associated with physiological youth is considerably but not entirely accounted for by size changes in the cells.

G20. Oxygen Consumption by Bacterial Cultures. C. P. HEGARTY, Cornell University.

The consumption of molecular oxygen by bacteria was measured by cultivating the organisms in a glass container completely filled with a medium saturated with air at 37°. After growth was established, the gases were removed from the culture by heat and vacuum, and analyzed in a modified Haldane apparatus. Controls were held for the same time as the culture to determine oxygen consumption by the medium. The difference was considered to be the oxygen consumed by the culture.

It was found that all of the bacteria tested removed all of the dissolved oxygen (about 2 to 2.5 cc. per liter) in about 24 hours at 37°. This included several species of *Streptococcus* and *Lactobacillus*, some obligate and some facultative aerobes, and also *Clostridium sporogenes*. The rate of utilization of oxygen varies greatly. *Pseudomonas fluorescens* used more than 0.8×10^{-10} mgm. of oxygen per cell per hour, while cultures of *Streptococcus lactis* consumed less than 0.075×10^{-10} mgm. With the latter, the sugar consumption was about 100 times as large as that of oxygen.

Considerable differences were found in the amount of CO_2 produced by the various streptococci and lactobacilli. This method has also been used to determine small amounts of hydrogen or nitrogen gas liberated by bacteria.

G21. Factors Controlling Pigment Production by Mycobacterium phlei. MARY A. INGRAHAM, Department of Agricultural Chemistry, University of Wisconsin.

Mycobacterium phlei may contain as many as 10 carotenoid pigments including alpha carotene, beta carotene, kryptoxanthene, and esters of lutein, zeaxanthene and azafrin. The effect of a number of factors on gross pigmentation has been studied in some detail. On a glucose-asparagin medium the pigment content of the cells was relatively low, but additions of simple alcohols, glycols or glycerol markedly enhanced pigmentation. The effect of a number of sugars, sugar alcohols, acids, aldehydes, fats and miscellaneous compounds including phytol, beta-

ionone and a vitamin A concentrate was observed. All failed to improve pigmentation.

On the glucose-asparagin medium, without the addition of other carbon compounds, it was possible to vary the pigment content of the cells within wide limits. Cells remained white as long as the medium was kept alkaline (above pH 8.0). The presence of an excess of potassium or phosphate, or ferric or cupric ions also tended to prevent the accumulation of pigments. Noteworthy was the fact that by limiting the potassium content of the medium it was possible to check growth without checking glucolysis. Another interesting observation was the fact that ferric and cupric ions were adsorbed rapidly and quantitatively on the surfaces of the bacterial cells. Ethylene, salts of selenium, sodium, lithium, calcium, and magnesium, the oxidation-reduction potential, the light intensity and the temperature of incubation were without specific effect upon pigmentation.

Evidence is being gathered from observations on individual pigments and from experiments with resting cells, as to whether any one factor operates through synthetic or destructive mechanisms, or whether under certain conditions an increase in pigment content may be a response to physiological requirements of the organism. Until facts of this character are known, it is difficult to draw conclusions as to the function of the carotenoid pigments in bacterial metabolism.

G22. A comparison of Nine Presumptive Test Media on One-Hundred Water Samples. MICHAEL A. FARRELL, The Pennsylvania State College.

Standard lactose broth, buffered lactose broth, brilliant green lactose bile broth, fuchsin lactose broth, methylene blue-brom cresol purple broth, crystal violet lactose broth, formate ricinoleate broth, MacConkey's peptone lactose bile broth and trypaflavine broth were planted with 100 water samples in order to determine the relative efficiency of these 9 media in the detection of the coli-aerogenes group in water.

The amount of water planted into each medium was five 10.0 cc. portions, two 1.0 cc. portions and two 0.1 cc. portions. The 10 cc. portions were planted into double strength media and the other two volumes into single strength media. The results on these 9 presumptive test media were then separately carried through confirmatory and completed tests according to Standard Methods Procedure.

The results of this study show that standard lactose broth is the most sensitive of these 9 media studied, in the detection of the coli-

aerogenes group. Forty-three per cent of all samples examined were found to be positive with this medium. Fuchsin lactose broth is second in finding approximately 42 per cent of all samples positive. The remaining media headed by brilliant green lactose bile broth, all showed less than 40 per cent of the samples positive.

This data taken in conjunction with other work involving pure culture studies of 23 colon organisms and 9 spore forming organisms responsible for false presumptive tests strongly indicates that fuchsin lactose broth on the whole is superior to standard lactose broth as a presumptive test medium.

G23. Further Observations on the Presumptive Test for Bacterium coli in Water Supplies. M. P. HORWOOD AND C. G. DUNN, Massachusettes Institute of Technology.

This investigation was conducted as part of a program initiated by the Committee on Standard Methods of Water Analysis of the American Public Health Association. Human fecal material and three polluted surface waters were used as the sources of *Bacterium coli*. The procedure employed consisted of isolating *Bacterium coli* in pure culture through the use of purple lactose agar plates. Each culture fermented lactose broth in 24 hours at 37°C. with the production of acid and gas, and on microscopic examination was demonstrated to be a Gram negative bacillus resembling *Bacterium coli*. One-half cubic centimeter of the 24 hour positive lactose broth culture made from a typical looking *Bacterium coli* colony fished from purple lactose agar was then introduced into 99 cc. of sterile Butterfield buffered dilution water and stored at 45°C. until the following day. At the same time, dilutions containing 1:1,000 to 1:100,000,000 of the bacterial suspension in lactose broth were prepared and examined in triplicate in lactose broth at 37°C. for 24 hours to determine the titer of the original 24 hour lactose broth culture. The highest dilution in which all three triplicate tubes gave positive gas readings was taken as the lowest dilution to be used in the comparative productivity tests. The presumptive test media used for the comparative productivity tests included standard lactose broth, buffered lactose broth, brilliant green lactose peptone bile, 2 per cent, crystal violet lactose broth, formate-ricinoleate broth, fuchsin lactose broth and methylene blue-brom cresol purple lactose broth.

In the comparative productivity tests, 15 tubes of each medium were employed for each of the three dilutions used. All tubes were read

after 24 and after 48 hours at 37°C. Any appearance of gas was recorded as positive. The results were as follows: Standard lactose broth showed the greatest number of positive tests and formate-ricinoleate broth, the smallest number. Crystal violet lactose broth also yielded unsatisfactory results. The other presumptive test media, i.e., buffered lactose broth, brilliant green lactose peptone bile, 2 per cent, fuchsin lactose broth and methylene blue-brom cresol purple lactose broth, gave satisfactory but not equivalent results when compared with standard lactose broth.

G24. The Fermentation of Acetyl-Methyl-Carbinol by the Escherichia-Aerobacter Group and Its Significance in the Voges-Proskauer Reaction. RALPH P. TITTSLER, University of Rochester School of Medicine and Dentistry.

The ability to ferment acetyl-methyl-carbinol has been determined for 174 cultures of the *Escherichia-Aerobacter* group and the results compared with the V-P reactions of *Aerobacter* cultures grown for varied periods of time in Clark and Lubs' medium.

The results may be stated briefly as follows: acid was produced by 17 of the 34 strains of *Aerobacter aerogenes* and by 20 of the 32 strains of *Aerobacter oxytocum*; but not by *Aerobacter cloacae*, 22 strains; *Aerobacter levans*, 1 strain; the *Escherichia* genus, 50 strains and the *Escherichia-Aerobacter* "intermediates," 35 strains. A reciprocal relationship was shown between the fermentation of acetyl-methyl-carbinol in nutrient broth and the ability to grow in Koser's synthetic medium, when acetyl-methyl-carbinol was substituted for sodium citrate as the sole source of carbon. Another reciprocal relationship was found between the ability to ferment acetyl-methyl-carbinol and the failure to obtain positive V-P reactions in *Aerobacter* cultures in Clark and Lubs' medium which had been incubated for more than from 2 to 4 days.

Thus, the frequent failure to obtain positive V-P reactions in old cultures of some *Aerobacter aerogenes* and *Aerobacter oxytocum* strains is due to the actual fermentation of acetyl-methyl-carbinol.

G25. Escherichia-Aerobacter "Intermediates" from Human Feces. PHILIP L. CARPENTER AND MACDONALD FULTON, Brown University and University of Wisconsin.

Escherichia-Aerobacter "intermediates" have been found in 13.5 per cent of 466 samples of human feces examined. Such strains were generally accompanied by typical *Escherichia* and *Aerobacter* organisms.

The 135 strains studied could be grouped into about 30 types by use of H_2S production, action on milk, and fermentation of carbohydrates. Only one strain corresponded with any of the recently proposed species of *Citrobacter*. Fermentation of cellobiose correlated most perfectly with the citrate, M.R. and V.P. tests, by which the organisms were classified as intermediate strains. In the other tests over 90 per cent of the organisms gave reactions typical of neither *Escherichia* nor *Aerobacter*.

The present confusion in the classification of such "intermediates" suggests two solutions: (1) that the genus *Citrobacter* be redefined to include all non-sporeforming aerobic bacteria which ferment dextrose and lactose with the formation of acid and gas, and are methyl red positive, Voges-Proskauer negative, and utilize citrate as a source of carbon; or (2) that the "intermediates" be considered new species or variants of already existing species in the genus *Escherichia*, which they resemble in the methyl red and Voges-Proskauer reactions. The latter solution seems most logical since little practical evidence has as yet appeared which justifies formation of a new genus.

G26. The Nature of the Change from Slow to Rapid Lactose Utilization by a Member of the Colon Intermediate Group. C. K. Wadsworth and E. R. Hitchner, Department of Bacteriology, University of Maine, Orono.

A slow lactose-fermenting member of the colon intermediate group upon being aged in lactose nutrient media acquired the ability to ferment lactose rapidly. The two strains, slow and rapid, could be very sharply distinguished from each other by the titratable acidity formed by cultures in nutrient broth containing one per cent lactose.

In a study of the change in ratio of numbers of the slow fermenters to rapid fermenting organisms occurring during the aging process the following procedure was adopted: The slow lactose-fermenting strain was inoculated into one per cent lactose nutrient broth. One cubic centimeter was withdrawn at the end of 1, 2, 3, and 4 days and plated out on nutrient agar. Fifty colonies were picked from each plating, inoculated into one per cent lactose nutrient broth, and the acidity produced in 24 hours was determined by titration with 0.005 N NaOH.

The subcultures made after 1, 2, and 3 days aging produced practically no greater acidity in 24 hours than did slow controls which had never been grown in lactose media.

Sixty per cent of the subcultures made after 4 days' aging produced

an acidity as high as did rapid controls which had been trained to utilize lactose rapidly.

It is concluded that the acquisition by the slow lactose-fermenting strain of the ability to utilize lactose rapidly is abrupt rather than gradual.

G27. Cultural Characters, Relationships, Significance and Occurrence of the Coli-Aerogenes Intermediates, with Particular Reference to Feces, Fresh and Stored at Various Temperatures. LELAND W. PARR, Department of Bacteriology, Hygiene and Preventive Medicine, School of Medicine, The George Washington University.

A heterogeneous group of Gram negative, lactose fermenting, non-sporing bacilli,—unofficially the Genus *Citrobacter*, to the laboratorian the coli-aerogenes intermediates,—has in recent years commanded attention of workers in the colon group field. Intermediates are colon group organisms possessing one or more essential coli characters and one or more of those attributed to aerogenes. In addition they are reputed to produce trimethylene glycol and some are strongly hydrogen sulphide positive on test.

Cultural study has been made of a collection of more than 100 intermediate strains deriving from soil, water, milk, foodstuffs, shellfish, occasional pathology, and feces, both fresh and stored. In addition data are presented for all intermediates encountered in an extended study of the colon group flora of normal human feces examined fresh and as stored suspensions held at various temperatures.

Cultural characteristics are presented on the framework of the "IMVIC reaction," i.e., indol, methyl red, Voges-Proskauer and citrate utilization results, rather than on the basis of primary classification based on fermentation tests. The data include, however, the usual reactions employed in studies of organisms of the colon group.

The relationships of intermediates to other organisms of the group are discussed.

Intermediates are rare in fresh feces except at times of establishment or reestablishment of the colon group. They are more common in cold storage specimens than in duplicates held at body temperature. They are probably typical of intestinal levels not readily sampled in fecal examinations or are even not adapted to life in the body. Their occasional occurrence in fresh feces in large numbers, however, makes it unsafe to denominate them for sanitary purposes as non-fecal.

G28. Hydrogen Sulfide Production as a Differential Test in the Colon Group. REESE VAUGHN AND MAX LEVINE, Iowa State College, Ames.

In a previous communication it was reported that in a medium containing 2.0 per cent proteose peptone (Difco), 1.5 per cent agar and 0.1 per cent K_2HPO_4 (anhydrous) with 0.05 per cent ferric citrate as an indicator, production of hydrogen sulfide was almost perfectly correlated with the citrate positive, methyl red positive and V.P. negative strains of the colon group. Modifications of the medium, such as changing the indicator or concentration and type of peptone or addition of cysteine as a sulphur source, which have appeared in the literature were employed in a study of 697 strains of the coli-aerogenes group comprising 229 *Escherichia*, 299 *Aerobacter* (105 *Aerobacter cloacae* and 194 *Aerobacter aerogenes* strains) and 169 "intermediate" strains.

The concentration of peptone was not a significant factor.

In the presence of cysteine the production of hydrogen sulfide had no differential value, as practically all strains were positive.

Reducing the concentration of agar markedly reduced the differential value of hydrogen sulfide production. With a concentration of 1.5 per cent agar, 74.0 per cent of the "intermediate" strains produced hydrogen sulfide whereas only 1.8 per cent of the *Escherichia* strains and none of the *Aerobacter cloacae* or *Aerobacter aerogenes* strains were able to produce the phenomenon after 48 hours at 37°C.

When the concentration of agar was reduced to 0.5 per cent, 74.0 per cent of the "intermediate" and 37.6 per cent of the *Escherichia* strains produced hydrogen sulfide while only 0.95 per cent of the *Aerobacter cloacae* and none of the *Aerobacter aerogenes* strains produced the reaction after 48 hours at 37°C.

With no agar present in the medium there were 93.5 per cent of the *Escherichia* strains, 91.1 per cent of the "intermediate" strains, 43.8 per cent of the *Aerobacter cloacae* and 74.7 per cent of the *Aerobacter aerogenes* strains which produced hydrogen sulfide from proteose peptone after 48 hours at 37°C.

An explanation of the influence of the inert agar has not been definitely established.

G29. Reaction of Escherichia, Aerobacter and Citrobacter Strains in Boric Acid and Hexamine Media. M. T. BARTRAM AND L. A. BLACK. Department of Bacteriology, University of Maryland.

Week old agar slant cultures and 24 hour broth cultures of strains of the *Escherichia-Aerobacter* group, isolated from raw and pasteurized milk, were used to inoculate a medium containing 0.325 per cent boric acid, as suggested by Vaughn and Levine, and a hexamine medium proposed by Wilson. The boric acid medium was incubated at 37°C. and at 43–44°C. (temperature of the medium) while the hexamine medium was incubated only at 37°C.

Of the 170 strains of *Escherichia* studied, 94.1 per cent produced acid and gas in boric acid medium after 48 hours at 37°C., 77.2 per cent produced acid and gas in the same medium at 43°C. and 94.1 per cent grew with the production of acid in hexamine medium. The 49 strains of *Aerobacter* were inhibited by the boric acid medium, 27.4 per cent producing acid and gas and 41 per cent producing acid only at 37°C.; at 43°C. only 6.12 per cent produced acid and gas and 2.1 per cent produced acid only, while in the hexamine medium 69.4 per cent gave positive results. At 37°C. 18.8 per cent of the 67 strains of *Citrobacter* produced acid and gas, and 49.4 per cent showed acid formation; at 43°C. neither acid nor gas was formed, while 65.2 per cent grew in hexamine medium. There was no difference in the results obtained when the cultures used for inoculation were week old agar slants and when 24 hour broth cultures were used.

The results with boric acid agree well with those obtained by Levine and his associates, with the exception that a considerably smaller percentage of strains classified as *Escherichia* developed at 43°C. compared to 37°C. It is apparent that hexamine in the concentration used did not exert sufficient inhibitive action on *Aerobacter* and *Citrobacter* to permit the differentiation of these genera.

G30. Distribution of Escherichia coli in Cold-Blooded Animals. A. A. Hajna and C. A. Perry, Bureau of Bacteriology, Maryland State Department of Health, Baltimore.

A comparative study of strains of *Escherichia coli* of cold-blooded animal origin was made to see if, by use of certain differential media, differences in the strains would be noted in relation to the source of the animals and the extent of pollution of areas.

All of the organisms were isolated from the intestinal contents of 29 cold-blooded animals (4 perch, one shad, one chub, one carp, one eel, 6 catfish, one garfish and 14 crabs) in standard lactose broth. Of 60 coli-like strains isolated on Levine's eosin-methylene-blue plates,

35 (58.3 per cent) fermented cellobiose (acid and gas), utilized citrate (Koser's medium) and failed to produce gas from dextrose at 46°C. (modified Eijkman medium of Perry and Hajna), or to produce indol. The animals were from unpolluted to slightly polluted areas.

The remaining 25 strains failed to ferment cellobiose or to utilize citrate as source of carbon, produced indol and fermented dextrose at 46°C. with gas. The majority of the strains were from the 6 catfish and a few crabs derived from areas moderately polluted. These latter strains of *Escherichia coli* correspond to those from warm-blooded animals.

This study indicates that *Escherichia coli* is not a natural inhabitant of the intestinal tract of cold-blooded animals but its presence results from the drinking of polluted water by these animals. There is perhaps some increase in numbers if water temperature is comparatively high.

G31. The Effect of Sodium Selenite on the Growth of Bacteria and Its Use As the Basis for a New Enrichment Medium for the Isolation of Typhoid Bacilli from Feces, Water, Milk, Etc. EINAR LEIFSON, Department of Pathology, Johns Hopkins University.

Sodium selenite has remarkable differentially inhibiting effects on the growth of various bacteria. Typhoid, cholera, proteus, and pyocyaneus bacilli can grow on infusion media at pH 7.0 containing as much as 1 per cent sodium selenite. Most other bacteria are much more sensitive. Colon bacilli are considerably inhibited at concentrations of 0.2 per cent and dysentery bacilli will not grow at 0.1 per cent concentration. Typhoid and dysentery bacilli are thus readily differentiated by selenite media. Other bacilli which seem to be differentiable by means of selenite media include alkaligenes and bronchisepticum and various types of *Brucella*. Work with the *Brucella* group is not yet completed. Liquid media with sodium selenite, as enrichment media for the isolation of typhoid bacilli, have been developed which show every promise of being much superior to those in use at present. For the isolation of typhoid bacilli from milk, 0.1 to 0.2 per cent of sodium selenite plus 1 per cent peptone at pH 7.0 is added to the milk which is then incubated overnight. Streaking is then made on desoxycholate-citrate agar. For the isolation of typhoid bacilli from feces, sewage, or water the medium has the tentative formula: sodium selenite (anhydrous) 0.3 per cent, peptone 0.5 per cent, sodium phosphate (anhydrous) 1 per cent, lactose 0.4 per cent, at pH 7.0.

Any quantity of feces, sewage, or water may be tested by adding the above ingredients in the proper concentrations. After incubation overnight the culture is streaked on desoxycholate-citrate plates and if typhoid bacilli are present they will appear in large numbers (usually) as readily recognized translucent colonies.

G32. An Apparatus for Collecting Bacteria in the Stratosphere. L. A. Rogers and Fred C. Meier, Bureau of Dairy Industry and Division of Coöperative Extension, U. S. Department of Agriculture, Washington, D. C.

On the invitation of the National Geographic Society an apparatus was constructed and with parachute attached was dropped from the stratosphere balloon at an altitude of about 14 miles.

The collector consisted of a glass cylinder 4 inches in diameter through which the air flowed as the apparatus fell. A baffle directed the air against the sides of the cylinder which were coated with 60 per cent glycerine. Dampers, operated by atmospheric pressure, were set to close the cylinder at an altitude of about 35,000 feet.

For developing colonies the inside wall of the cylinder is covered with nutrient agar using the technique of the Esmarch tube.

G33. Protein-Sparing Action of Carbohydrates in Relation to Anaerobic Identification. Robb Spalding Spray and Alfred Reeves Stanley, Medical School, West Virginia University, Morgantown.

Considerable study has been made of the effect of fermentable carbohydrates on proteolysis by the aerobic bacteria. Little, if any, similar work has been reported for the anaerobes, in spite of the fact that glucose is commonly added to media as an available source of oxygen. The older methods involve complex determination of NH_3 and analogous compounds. This appears impractical as pertains to the anaerobes. We have employed, instead, a series of directly visible colorimetric reactions with interesting results.

Our tests, in general, support the hypothesis of the "protein-sparing" action of fermentable carbohydrates, but certain individual peculiarities of reaction tend to confuse its application in specific instances. The question is one of great importance in its connection with the routine diagnostic tests for indol, nitrate reduction, H_2S blackening and gelatin liquefaction as used in identification of anaerobes.

G34. III. Further Development of Semisolid Media in Cultivation and Identification of Sporulating Anaerobes. ROBB SPALDING SPRAY, Medical School, West Virginia University, Morgantown.

For the past three years the writer has presented before this Society certain principles and details of methods of cultivation and identification of the sporulating anaerobes by use of semisolid and other diagnostic mediums.

A grant of the National Research Council has made possible the extension of this study to over 300 cultures. The Tentative Key, presented last year, has been amplified and fortified by the study of at least 10 strains (where available), and in certain instances as many as 44 strains of *Clostridium Welchii* and 87 *vibrion septique* were used.

While it is believed that the reactions in this Tentative Key are correct, its final presentation is being withheld in order to carry the work over a number of important species.

The formulas of these mediums have been slightly altered in an attempt at simplification and improvement of growth and of strength of certain reactions, such as indol and nitrite.

These methods make possible simultaneous comparative study of many strains—in one instance 64 strains of *vibrion septique* were studied at one time. Such is impracticable by the older methods.

G35. Preserving Anaerobes by Desiccation. ALDEN F. ROE, The George Washington University School of Medicine.

In the fall of 1933 a method for desiccating anaerobes was devised. It included some of Swift's as well as Brown's technique for aerobes and briefly outlined involved: (*a*) concentration of cells of very young cultures, (*b*) resuspension in a minimum of sterile serum, (*c*) desiccation on strips of filter paper in a vacuum over P_2O_5 while frozen, (*d*) sealing strips in a vacuum in individual tubes with $CaCl_2$ and (*e*) storing at a low temperature.

This method of desiccation was applied to 16 species of the more important anaerobes, with several variants, totaling 212 strains. As a control on the method 6 of the more delicate aerobes were similarly desiccated.

That anaerobes afforded a more difficult task in desiccation than aerobes was evidenced by the fact that 14 of the 212 strains were found non-viable on testing in liver broth immediately after desiccation. A

retrial on these strains however brought them through in a viable state. All strains were also tested for the presence of spores (80° for 30 minutes) and aerobe contaminants.

After remaining one year in the desiccated state, these strains were tested for viability, spores and aerobic contaminants. In October of this year they were again tested but due to shortage in some of the series, only 203 strains were inspected.

Starting with 100 per cent of the strains viable after desiccation, after 1 year 98 per cent were still viable and after 2 years 96 per cent were still viable. No change in type of growth in broth or gas production was noted.

Originally the spores were found present in 49 per cent of the strains which after 1 year was reduced to 27 per cent and after 2 years to 26 per cent.

The extensive open technique involved in manipulation apparently introduced an appreciable number of aerobic contaminants, there being 9 per cent of the cultures involved originally as well as after 1 year and 13 per cent in the two year test.

The desiccated aerobe controls after the 1 and 2 year period were all still viable.

It appears from the data thus far obtained that desiccation can be satisfactorily applied to anaerobes for maintaining stock cultures and that certain advantages are obtained over culturing as to assuring longer viability and in fixing characteristics of the culture.

G36. The Preparation and Properties of Silicic Acid Jellies for the Pure Culture Isolation of Bacteria. JOHN H. HANKS AND R. W. WEINTRAUB, George Washington University Medical School.

Theoretical advantages from the use of silica gel as a specialized substitute for organic gels have not been realized, largely as the result of an erroneous assumption that inorganic gels are inert in a medium. Studies of the influence of time, temperature, dialysis and autoclaving on the pH, nutritive value and rigidity of silica gel have shown that each step in the preparation of the medium is attended by hitherto unsuspected changes.

By the incorporation of color indicators and nutrient salts in the sodium silicate (6 per cent) and the HCl (approximately 0.5 N) solutions and by combining these reagents until the proper pH was obtained, the nutrients, the hardening time and the pH of the gel mixtures were standardized and reproduced from batch to batch. Time

for adjusting the pH and for the distribution of large batches of the mixture into petri dishes was secured by using chilled reagents.

Gels thus prepared at pH 7.0, without nutrients, alkalinized slowly on standing and rapidly on autoclaving to a pH of 8.1. During dialysis in 10 times their volume of distilled or tap water, both the gels and the dialyzing fluid became alkaline; no equilibrium was reached, and the gels were peptized in the autoclave. Dialysis in nutrient salt solution of pH 7.4 caused initial alkalinization of the gels to pH 7.8 and acidification of the solution. After 20 hours at 40°C., equilibrium was indicated by stabilization of the pH curves at 7.6 and 7.4 for the gel and the fluid, respectively. These gels withstood autoclaving at 15 pounds for 15 minutes. Through loss of ammonia, the pH fell to 7.2.

Gels prepared with ammonium salts incorporated in the mixtures alkalinized only to pH 7.5 on standing and autoclaving. They dialyzed to a final pH of 7.6 with the solution at 7.4. These gels withstood autoclaving and had a final pH of 7.3.

Most effective control of the pH was secured with 4 times the usual concentration of ammonium salt in the original mixture and by dialyzing against the salt solution minus the ammonia. The pH shift was minimized and final sterilization was carried out at pH 7.6 without appreciable change in the H-ion concentration.

G37. Recent Developments in the Standardization of Basic Fuchsin, Brilliant Green, Crystal Violet and Methylene Blue. H. J. Conn, New York State Agricultural Experiment Station, Geneva.

This is a progress report on the work being carried on in coöperation between the Commission on Standardization of Biological Stains and the American Public Health Association. The 4 dyes here discussed have been given special attention, not as stains, but as indicators or bacteriostatic reagents.

Basic fuchsin, to be satisfactory for the bacteriologist, must not only be a good stain but must give good results in two or three culture media, each of which presents a problem of its own. Brilliant green should prove satisfactory in the brilliant green bile medium, permitting the growth of the colon group but preventing that of spore-formers, when employed in the dilution called for by present standard methods. Crystal violet should show similar properties in the way of bacteriostatic action.

Methylene blue, on the other hand, has of recent years been given

more study as to its behavior in the reductase test for milk than as a stain or as a bacteriostatic reagent. Recent coöperative work makes it look as though the dye were now nearly standardized for this very special use.

G38. A Single Cell Study of the Smooth and Rough Strains of Staphylococcus aureus. RACHEL E. HOFFSTADT AND GUY P. YOUMANS, University of Washington, Seattle.

Single celled cultures of the smooth and rough strains of *Staphylococcus aureus* were studied for dissociation, virulence and immunological reactions. The cultures were grown in lithium chloride broth and plain broth. All yellow strains studied differed from the original yellow strain in that they fermented glycerine. Both yellow strains dissociated, but the white did not. Only one variant obtained showed the cultural characteristics of any others previously reported by us.

G39. Fermentation Products of the S and R Forms of Yeasts. F. W. FABIAN AND L. J. WICKERHAM, Michigan State College, East Lansing.

A detailed study of the products of fermentation of the S and R forms of 4 yeasts, *Saccharomyces cerevisiae* Hansen, Saaz strain, *Saccharomyces aceris-sacchari* Fabian and Hall, *Pichia alcoholophila* Klöcker and *Willia anomala* Saito was made in three different media, cider, malt extract broth and a synthetic medium. The results showed that in some species there is a marked difference in the fermentation properties of the S and R forms, while in other species there is little or no difference. The S forms produced alcohol more rapidly and in greater amount than did the R forms. The production of volatile acids was extremely variable. The amount produced varied with the different media, with the species of yeast, and with the S and R forms of the different species.

Ester production by the S and R forms of the 4 yeasts studied did not show on the whole as great a variation as did the volatile acid production. There were some exceptions to this as for example in the case of *S. cerevisiae* where the S form produced practically no esters in cider and malt extract broth while the R form produced a large amount of esters. The conditions under which the yeasts were grown likewise influenced ester production. Sufficient oxygen to maintain normal growth produced the largest amount of esters. When there was an abundance or insufficient oxygen present, there were no esters produced. Esters produced by the yeasts did not appear in appreciable

quantities until 35 to 45 days after inoculation. Shortly after the esters appeared there was a corresponding decrease in alcohol and volatile acids. However, experiments, in which the amounts of alcohol and volatile acids were maintained in the same concentration as that produced in the fermented media, failed to show ester production. Likewise, the addition of dried ground yeast cells to the above solutions did not influence ester production. It was, therefore, concluded that within the time limit of these experiments the esters were produced within the living cells during the process of fermentation rather than by a simple chemical reaction between the alcohol and acids or by an endoenzyme liberated by the autolyzed yeast cells.

The three media used affected the stability of the yeasts differently. The S and R forms of all 4 species were the most stable in the synthetic medium. In this medium there was no tendency for the S form to change to the R form or vice versa. There was a greater tendency for them to change in cider while in malt extract broth they were the least stable.

G40. Dissociation of the Pneumococcus into a Stabilized Diphtheroid Bacillus. LAWRENCE E. SHINN, PHILIP B. HADLEY AND RALPH R. MELLON, The Western Pennsylvania Hospital Institute of Pathology. Abstract withdrawn.

G41. A Theory of Bacterial Variation, Variation in Serratia marcescens. G. B. REED, Queen's University, Kingston, Canada.

In *Serratia marcescens* and some other species, variation has been shown to occur in any one of three sets of characteristics (colony structure, capsulation, pigmentation) independent of variation in the other two sets. These results support the hypothesis that variation results from chance failure of one or more genes to divide or from division and failure to segregate in cell division.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

M1. Factors of Resistance in Experimental Poliomyelitis. N. PAUL HUDSON, EDWIN H. LENNETTE AND FRANCIS B. GORDON, Department of Bacteriology, Ohio State University and Department of Hygiene and Bacteriology, University of Chicago.

Experiments were conducted in poliomyelitis attempting to analyze factors that may possibly influence resistance to the disease. From the data derived, it appears that (1) there is an element of variable

resistance in the nasopharyngeal mucosa that was experimentally alterable; (2) the normal intestinal mucosa was impervious to the entrance of poliomyelitis virus; (3) removal of the spleen as a large depot of reticulo-endothelial cells seemed to increase in some animals the susceptibility to intravenous virus, when the splenectomy preceded the virus injections, and not when splenectomy was done after virus was injected; (4) the virus taken up by splenic cells was demonstrable when the spleen was freed of antibodies by perfusion, but antibodies were not demonstrable in the cells of perfused spleens; (5) the occurrence of the poliocidal property in the blood serum was not parallel to, and was found far more frequently than, resistance to intranasal instillation of virus; (6) breaking the blood-CNS barrier by cerebral starch injections induced infections after intravenous administration of doses noninfective for controls; (7) the observation of others that the olfactory tract is the avenue of entry of the virus was confirmed by failure to infect 5 monkeys by this route after the tracts were cut; the same animals resisted large intravenous doses of virus fatal to 4 of 5 controls.

It may be concluded from these necessarily limited observations that in experimental poliomyelitis, the nasopharynx offers some resistance to infection while the olfactory tract itself is the natural essential portal of entry. The spleen has some part in the systemic resistance to the disease, in its cells taking up the virus, but how the virus is disposed of is not clear. No evidence was found to show where the poliocidal antibodies are formed. The presence of antibodies in the serum is not a direct measure of the animal's resistance to experimental administration of the virus intranasally. The central nervous system seems to be the essential site of pathogenesis and susceptibility to the natural mode of infection. Measures designed to protect against poliomyelitis should take this into account.

M2. Failure to Establish Poliomyelitis Virus in Normal Mice and Following Extirpation of the Thyroid Gland and the Testes. RANDALL L. THOMPSON AND EARL B. MCKINLEY, Department of Bacteriology, Hygiene and Preventive Medicine, School of Medicine, George Washington University.

Certain investigators have attached considerable importance to the rôle of endocrine glands in resistance to poliomyelitis infection. Brodie has reported the establishment of infection with poliomyelitis virus in x-ray treated mice. An experiment was designed to determine if mice

might not be susceptible to this virus after the removal of one or more endocrine organs. Forty-six young male mice were divided into 4 groups: (*a*) thyroidectomized, (*b*) castrated, (*c*) thyroidectomized and castrated, and (*d*) normal animals. Each mouse received an intracerebral injection of infected monkey cord during the eighteenth week of life. Five animals died during an observation period of 47 days; sections of the brains of these animals showed no evidence of inflammation.

M3. Attempts at Vitamin C Therapy in Experimental Poliomyelitis.
CLAUS W. JUNGEBLUT, Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York.

We have already shown that vitamin C (ascorbic acid) when combined with poliomyelitis virus *in vitro* brings about inactivation of the infectious agent as determined by the intracerebral injection of the mixture into Rhesus monkeys. A striking feature of this phenomenon was the existence of a fairly narrow quantitative range within which vitamin C seemed to be most effective, the optimal doses ranging between 10 and 1 mgm.

The fact that such extraordinarily small amounts of vitamin C are capable of inactivating the virus of poliomyelitis *in vitro* is not only of theoretical interest but might be of practical importance. Since amounts of vitamin C within the virucidal range are normally present in the central nervous system of man, the question arises whether or not the natural insusceptibility to poliomyelitis of the great majority of humans is not related with the presence of this substance in nervous tissues. On the other hand, we have presented evidence that the exceptional susceptibility to poliomyelitis of certain children and adults depends upon some endocrine disturbance. This might well be correlated with a faulty vitamin C metabolism.

In view of these considerations experiments were undertaken to investigate whether vitamin C possessed any therapeutic properties in experimental poliomyelitis. Our present work comprises 3 groups of monkeys which received various doses of vitamin C by the subcutaneous route—beginning with the day of infection and repeated daily for a period of two and one-half weeks—and a fourth group which was left untreated for control purposes. All animals were infected with a uniform dose of 0.1 cc. of a 10 per cent virus suspension intracerebrally. All 4 monkeys treated with 5 mgm. of vitamin C survived without showing any symptoms of poliomyelitis. The next best result was obtained

with 16 animals treated with from 10 to 50 mgm., two surviving without paralytic symptoms, three developing atypical poliomyelitis after greatly prolonged incubation periods (16 to 21 days), and the remainder developing typical paralysis. All 9 animals which had received the maximal doses (700 to 100 mgm.) showed typical poliomyelitis, as did all of the 10 controls.

Although it is premature to draw any definite conclusions from this preliminary report there seems to be a strong probability that vitamin C when injected in the proper dose possesses distinct therapeutic power in experimental poliomyelitis.

M4. Protective Action of Certain Chemicals Against Infection with Poliomyelitis Virus by the Nasal Route. A. B. SABIN, P. K. OLITSKY AND H. R. COX, Laboratories of The Rockefeller Institute for Medical Research.

Olitsky and Cox (1934) showed for the first time that mice which received a number of nasal instillations of tannic acid were rendered resistant to subsequent infection with equine encephalomyelitis virus by the nasal route. Shortly thereafter, Armstrong (1935) reported a similar effect of sodium alum in the prevention of nasal infection of mice with the virus of St. Louis encephalitis and, while the present investigation was in progress, Armstrong and Harrison reported similar experiments on the prevention of poliomyelitis in monkeys.

The purpose of the present investigation was to determine: (1) whether nasal instillation of tannic acid or sodium alum had any effect on the susceptibility of monkeys to infection with poliomyelitis by the nasal route, (2) the concentration of the chemicals required to produce the optimum effect, (3) the time required for the refractory state to be induced, (4) the duration of the refractory state, (5) whether the refractory state could be maintained over a period of weeks by daily instillations, (6) the action of these chemicals upon the virus, (7) whether specific antiviral immunity developed in the refractory animals, and (8) the effect of these chemicals in the nose of man.

Typical poliomyelitis was induced in 26 of 34 untreated monkeys by the instillation on two occasions, 48 hours apart, of 1 cc. of a 10 per cent suspension of poliomyelitic cords into each nostril. The majority of monkeys treated with either sodium alum or tannic acid for a number of days prior to the instillation of virus were distinctly resistant to poliomyelitis. Only 2 of 16 monkeys treated in that manner with 4 per cent sodium alum developed the disease, and these were in a group

which apparently received more than the average amount of virus. Most of the monkeys treated with 3 per cent alum also proved resistant, while 0.5 per cent and 2 per cent had no effect. Four per cent tannic acid, although used in a smaller series, was also effective, while 0.4 per cent and 0.8 per cent were not. Effective concentrations of alum or tannic acid mixed with the virus *in vitro* did not diminish its disease-producing capacity.

Treatment with alum for at least a few days prior to infection was necessary to induce resistance. The resistance could be maintained over a period of several weeks by one daily instillation of the chemical; omitting the treatment for 48 hours diminished the number of monkeys that were resistant. Since in the resistant monkeys no treatment was given after the last dose of virus, it would appear that the virus of poliomyelitis does not survive long in them. Refractory monkeys showed neither fever nor any other signs which could be attributed to poliomyelitis and, when tested 1 to 2 months later for active immunity by intranasal administration of virus, were found to have none; their blood contained no antiviral bodies. Four per cent sodium alum was given intranasally to a number of volunteers and no untoward symptoms, other than slight local irritation and nasal discharge for a few hours following treatment, were observed.

Experimentally there is now sufficient basis for a trial in man of these chemicals in the prevention of poliomyelitis during epidemics.

M5. The Antibody Content of Acute Poliomyelitis Cases as Compared with Normal Individuals. MAURICE BRODIE, Department of Bacteriology, New York University Medical School.

The antibody content of a group of paralytic poliomyelitis cases was tested during the first week of the disease, and compared with that present in normal individuals. The presence and amount of antibody were compared for different age groups. It was found that acute polio cases usually had little or no antibody. Inasmuch as the level of antibody is decidedly lower in poliomyelitis cases than in the case of so-called normal individuals, it would seem that the lack of antiviral substance is one of the factors predisposing individuals to poliomyelitis.

Thus the presence of antibody appears to protect against the disease. This is further borne out from the fact that the incidence and degree of antibody show a gradual increase with age in the so-called normals. This is in keeping with the lower incidence of the disease in the higher age groups.

M6. The Immunological Relationship Between the Viruses of Human and Swine Influenza. THOMAS FRANCIS, JR., Hospital of The Rockefeller Institute for Medical Research.

The serum of experimental animals convalescent from infection with the virus of human or swine influenza contains antibodies which confer complete passive protection on mice against the homologous virus. After repeated inoculations with one virus, however, the immune zone is broadened so that the serum may then offer cross passive protection to mice against the heterologous virus as well. This effect is most definite in the serum of animals receiving repeated inoculations of human influenza virus. The possible relationship of these observations to immunity tests made with human sera against influenza virus will be discussed.

M7. The Susceptibility of Swine to the Virus of Human Influenza. RICHARD E. SHOPE, Rockefeller Institute for Medical Research, Princeton, N. J.

Swine inoculated intranasally with the virus of human influenza develop an ill-defined, mild and usually afebrile illness of short duration. At post mortem the anterior lobes of the lungs of such animals contain scant, scattered areas of lobular atelectasis. Transmission of the virus for five serial passages through two groups of swine has failed noticeably to enhance its activity for this species. Virus, pathogenic for mice, was demonstrated in the involved lung of each passage animal. The clinical and pathological picture presented by swine infected with the virus of human influenza is similar to that caused by the virus of swine influenza. If the virus is administered intranasally under ether narcosis a somewhat more severe illness is produced and the pulmonary lesions are more extensive. Swine inoculated intranasally with the virus of human influenza and a small amount of a culture of *Hemophilus influenzae suis* develop a febrile, prostrating illness similar to swine influenza. The pneumonia encountered at autopsy of such animals is similar, though somewhat less extensive as a rule, to that seen in swine influenza. Swine recovered from infection with the virus of human influenza are resistant to swine influenza. The sera of such animals, however, do not neutralize the virus of swine influenza.

M8. Interstitial Pneumonia in Mice. PERRIN H. LONG AND ELEANOR A. BLISS, Johns Hopkins University Medical School, Baltimore.

During the past year we have been able to transfer influenza virus directly from man to stock mice without employing an intermediate

passage through ferrets. These transfers were accomplished with both filtered and unfiltered rhinopharyngeal washings from individuals ill with human influenza. In each instance the infection was carried through several mouse passages and the resulting pathological lesions consisted of a typical interstitial pneumonia. Similar lesions, however, have been produced by the inoculation of pure cultures of various bacteria into our stock mice. As yet we have been unable to transfer cold virus from man to mice.

M9. The Action of Formolized Virus as a Preventive of Experimental Equine Encephalomyelitis in Guinea Pigs. HERALD R. COX AND PETER K. OLITSKY, Laboratories of The Rockefeller Institute for Medical Research.

In elaborating the observations of Shahan and Giltner on the immunizing action of formolized equine encephalomyelitis virus, we found that the virus in contact with 0.4 per cent formalin for 2 to 4 days at 22°C., followed by storage for 2 to 65 days at 4°C., immunized guinea pigs. Two 1 cc. subcutaneous injections at 7 days' interval induced resistance to 1,000 to 10,000 intracerebral lethal doses given 12 to 90 days later. Of 15 vaccines prepared, no active virus was demonstrated by intracerebral inoculation of over 700 mice and 30 guinea pigs, nor by combined intracerebral and intraperitoneal injection of guinea pigs with as much as 8 cc. Attempts to recover active virus from tissues of animals receiving massive doses of the vaccines were unsuccessful.

A comparative study of the immunizing capacity of active, untreated virus revealed that to obtain the same degree of resistance, it was necessary to inject subcutaneously 3,000 to 30,000 mouse intracerebral lethal units of the virus three times at 7 days' interval. Lesser amounts of this material failed to confer protection against the intracerebral test inoculation and larger amounts increased the danger of producing disease during the period of immunization.

It should be emphasized that these findings apply to the virus studied and not necessarily to other virus agents.

M10. Limited Neurotropic Character of St. Louis Encephalitis Virus in Susceptible Mice and its Transmission from Nose to Brain by the Olfactory Route. LESLIE T. WEBSTER AND ANNA D. CLOW, Laboratories of The Rockefeller Institute for Medical Research.

Virus injected directly into the brains of susceptible mice multiplies there and reaches a titre of 10^9 intracerebral lethal doses. It is also

found in the blood in small amounts following injection and preceding death. Virus injected intraperitoneally or subcutaneously circulates in the blood for several hours and survives in the spleen for days; however, it does not multiply in the brain and cause encephalitis unless overwhelming doses are injected or the brain is traumatized. Multiplication of virus in the spleen can be demonstrated by repeated intraperitoneal passages of virus containing spleen.

Virus dropped into the nose is demonstrable in the olfactory bulbs in 24 hours, in the piriform lobes in 24 to 48 hours, in the remainder of the brain at 3 days, and in the spinal cord in 4 days. In the brain, it reaches a titre of 10^9 in 6 days. Virus is not readily demonstrable in the blood but is present in the spleen after 48 hours. Lesions appear first in the ventral portions of the olfactory bulbs on the third day, next, in the piriform lobes on the fourth day, and in Ammon's horn on the fifth day. The initial lesion consists of perivascular and subpial accumulations of mononuclear cells, the second lesion is hyperplasia of the pial endothelium, and the third, necrosis of nerve cells. Thus, by the time encephalitis is recognized clinically in the nasally infected, highly susceptible mouse, virus has been present in the nervous tissue 5 days, lesions have been present 2 days, and the infection is far advanced and progressing rapidly.

M11. Cultural Characteristics of Aerobic Gram-Negative Bacilli Isolated from Genito-Urinary Infections. LESLIE A. SANDHOLZER, University of Rochester, School of Medicine and Dentistry.

A study has been made of the aerobic, Gram-negative bacilli in urinary infection. In all 530 cultures from 283 patients have been subjected to detailed taxonomic investigation with the aim of ascertaining whether or not there is any relationship between species or types and the clinical course of the infections. This paper deals only with the bacteriological findings.

The cultures were divided into 6 genera. *Escherichia* occurred in 83 per cent of the cultures (27 species or types); *Aerobacter* in 13 per cent (14 species or types); the remaining 4 per cent were distributed among *Salmonella*, *Proteus*, *Shigella* and *Pseudomonas*.

Hemolytic activity was determined on rabbit's blood-pork infusion agar in poured plates. While all of Brown's types of hemolysis were observed, the type was independent of genus or species.

Where several specimens were obtained from the same individuals over a period of months, the infecting organism remained constant as

to species and hemolysis; in other cases, either one or both of these factors varied. One patient had 9 different species from 3 genera showing every hemolytic type except *alpha prime*. These species were isolated at various times.

The correlation of this data with the clinical records is now being studied.

M12. Serum Studies in Experimental Meningococcus Infection:—Lack of Protection for Rabbits and Guinea Pigs. S. E. BRANHAM, A. M. PABST AND R. D. LILLIE, The National Institute of Health, Washington, D. C.

The authors have previously described the acute purulent leptomeningitis in rabbits and guinea pigs following intracisternal injection of virulent meningococci and of filtered broth cultures of some strains. The clinical and histopathological pictures produced by these two types of material were essentially identical. Here the effect of serums upon this meningitis was investigated.

With newly isolated cultures of meningococci of serological groups I–III and II, and filtrates from 6 day old broth cultures, the approximate minimum fatal dose was determined, and injections were made intracisternally.

The 20 polyvalent horse serums used had high aggutinin content. Six polyvalent horse antitoxins and 11 rabbit antitoxins were also included, besides several normal horse and rabbit serums. The effect of polyvalent serum and of antitoxin were studied both in animals given cultures and in those given the filtrates.

At first the inoculum and serum were mixed, and the mixture was given intracisternally. There was no protection by that method. Control experiments showed that horse serum in itself is toxic for rabbits and guinea pigs when given intracisternally, and that it may produce a fatal meningitis. That the preservative in the serum was not responsible for this effect was easily proved. The rabbit serums were not toxic, but they did not protect.

After this, the serums were given intravenously. They were not toxic by this route and much larger amounts could be given. The serum was given at various intervals, preceding, after, or at the same time as the intracisternal injection of culture or "toxin." In rabbits several injections of serum were frequently made.

There was no definite protection against the cultures or the "toxins" in either rabbits or guinea pigs. An occasional experiment gave prom-

ise, but consistent results were not obtained. On the whole the animals receiving the antitoxin showed a somewhat lower mortality than those receiving the polyvalent serum, but the difference was not great enough to be impressive.

Although clinical and histopathological meningitis may be readily produced in rabbits and guinea pigs by intracisternal injection of sufficiently virulent cultures and of some broth culture filtrates, meningitis in these animals does not seem to offer a suitable basis for the evaluation of antiserums.

M13. Cultivation of Cytoplasmic Inclusion Bodies Occurring in Human Throat Epithelial Tissues. JEAN BROADHURST, ROSAMOND A. LIMING, M. ESTELLE MACLEAN AND INEZ TAYLOR, Teachers College, New York City.

The cytoplasmic inclusion bodies occurring in the epithelial cells of the human throat have been successfully cultivated in embryonic chicken tissue (Goodpasture method), using 24-hour, one per cent acetic acid suspensions of material taken from a case of acute sore throat. In one egg series 8 serial transfers were thus obtained.

These inclusion bodies have also been transferred to monkeys from a human carrier as well as from a case of sore throat. Transfer from monkey to monkey by throat swabbing and by cage contact has also been secured, making in all a total of 7 positive monkeys which were selected from 29 monkeys previously found to be negative for these inclusion bodies.

Although these bodies stain with the common bacteriological stains they also stain pink with Giemsa's stain (especially after Zenker's fluid), as commonly reported for inclusion bodies. With the rabies stain (Sellers or Williams) a stippled appearance suggestive of the rabies bodies may be seen following decolorization by alcohol. Colored lantern slides will illustrate the contrast stains developed to help differentiate these bodies in such confusing situations as those met in egg tissue work and in the throats of monkeys due to the masses of food and extraneous material usually present.

M14. Studies in the Bacteriology and Immunology of Leprosy. HOWARD J. HENDERSON, JOSEPH D. ARONSON AND ESMOND R. LONG, The Henry Phipps Institute, Philadelphia.

Two apparently similar chromogenic acid- and alcohol-fast organisms were isolated from lepromas of different patients. One was isolated from the popliteal node of a rabbit inoculated intratesticularly with

tissue culture explants from a leproma. The second culture was isolated from the bone marrow of a frog inoculated in the dorsal sac with a suspension from another leproma. Colonies on Löwenstein's medium were soft, moist and orange colored. These cultures produced only local nodules when injected intracutaneously or intratesticularly into rabbits.

Of 16 bull frogs inoculated in the dorsal sac with suspensions from 16 lepromas acid-fast bacilli were demonstrable in the bone marrow or spleen in 10 frogs. No acid-fast bacilli were found in 24 uninoculated bull frogs of the same species.

Purified proteins were obtained from the two cultures, and compared with corresponding proteins from 5 stock strains of *Mycobacterium leprae*, from an avian, human and bovine type of *Mycobacterium tuberculosis*, from cultures isolated from tuberculosis of cold blooded animals and from *Mycobacterium butyricum*, *Mycobacterium smegmatis*, and *Mycobacterium phlei*. The proteins were prepared by growing these cultures on Long's medium, and filtering and precipitating the protein with trichloracetic acid according to the method of Seibert. Rabbits were immunized with the different proteins and the antigenic specificity was determined by means of the precipitin reaction.

A group relationship was found between the purified proteins. The proteins of the two lepromata cultures and their antisera reacted with the antiserum and protein obtained from *Mycobacterium leprae* Clegg, *Mycobacterium leprae murium* and *Mycobacterium phlei*. Walker's *Mycobacterium leprae* strain reacted only with homologous antiserum. Duval's nonchromogenic *Mycobacterium leprae* and the Levi-Kedrowsky strain differed antigenically from other strains of *Mycobacterium leprae*.

Intracutaneous tests on 158 leprous patients at Carville, La., and 263 nonleprous patients, with protein from *Mycobacterium leprae* Clegg and protein from one recently isolated leproma strain did not show sufficient specificity for practical diagnostic use.

M15. Increase in Toxicity Due to Action upon the Broth Constituents by Endo-enzymes of the Salmonella Bacteria. FRANCES L. KRAFT AND C. N. STARK, Cornell University, Ithaca, New York.

Gordon (1933, Thesis, Cornell University) in her studies of the production of heat-stable toxic substances by members of the *Salmonella* group of bacteria, showed a direct correlation between the death of the cells, the rise in pH, and increase in toxicity.

Further investigations have revealed that something in the nature

of a heat-stable endo-enzyme complex which, when released by a brief subjection to 100°C. or by natural autolytic processes, is capable of acting upon the broth to produce certain changes characterized by a gradual increase in pH, paralleled by an increase in toxicity. Results indicate that the heat-stable products so often referred to as "endotoxin" are not true endotoxins, but are split products of the culture medium resulting from the action on that medium by a heat-stable endo-enzyme, liberated during autolysis of the bacterial cells. This enzyme complex is not inactivated by 10 minutes of flowing steam temperature, but is inactivated by 20 minutes heating at 100°C. The toxic substances do not appear to be affected by being heated for 20 minutes or longer at 100°C.

M16. The Antigenic Relationships of Salmonella aertrycke var. binns. P. R. Edwards, Kentucky Agricultural Experiment Station.

The three known strains of the Binns type; namely, Greifswald, Timson and Binns, have been examined and all contain specific components characteristic of *Salmonella aertrycke*. The somatic antigens resemble those of *Salmonella abortivo-equinus* rather than those of *Salmonella aertrycke*. Instead of the antigenic formula IV, V, —, 1, 2, 3 that has been attributed to this type the formula IV, i, 1, 2, 3 should be substituted.

M17. Non-Sporulating Anaerobes in Ulcerative Colitis. G. M. Dack, Theodore E. Heinz and Lester R. Dragstedt, University of Chicago.

In a previous study some non-sporulating anaerobes were found to be the predominant species of bacteria in the diseased isolated colons of three patients with ulcerative colitis upon whom ileostomies had been performed (Dack, G. M., Heinz, T. E., and Dragstedt, L. R. Arch. Surgery, 1935, **31**: 225). In this study an attempt was made to cultivate these organisms from the ulcerated colons of 20 patients and from the normal colons of 6 patients. Agglutination and complement fixation tests with certain of these strains were made against a larger series of cases and controls. Attempts to reproduce ulcerative colitis in the isolated colon of 3 *Macacus rhesus* monkeys were made, using cultures or specimens removed from the diseased colons of patients.

The organisms were strictly anaerobic Gram negative pleomorphic rods which formed greyish raised colonies on blood agar plates. A greenish zone developed about the colonies when the plates were exposed to the air. Two types were encountered, a filamentous or fusi-

form-like, irregularly stained organism and a pleomorphic large rod with many long forms and numerous "ghost cells." These bacteria failed to grow on plain nutrient media. They were found in 15 of the 20 patients studied. Agglutinins and complement fixing bodies were found in low titre in the majority of patients with ulcerated colons and in only a few patients with no history of bowel pathology. Experiments to transmit ulcerative colitis to monkeys have been unsuccessful.

M18. Changes in Morphology of Hemophilus pertussis Grown under Varying Conditions and on Different Mediums. JOHN A. TOOMEY AND WM. S. TAKACS, Division of Contagious Diseases, City Hospital and Department of Pediatrics, Western Reserve University, Cleveland, Ohio.

Freshly isolated *Hemophilus pertussis* organisms grown on 20 to 25 per cent hemoglobin blood agar (Bordet) medium having a pH of 6.0, appear as small ovoid bacilli, at times nearly indistinguishable from coccoid bacteria. When these organisms are transferred every 4 days on the same medium they begin to elongate after 160 to 180 transplant generations, while their virulence and antigenic activity remain the same or slightly diminished. The organisms acquire a new quality, that of adhesiveness, not seen in freshly isolated cultures. A single passage through a guinea pig brings the organism back to its original ovoid form. It likewise regains what little virulence it has lost and loses its tendency to stick to the medium.

Organisms acclimated to veal brain agar medium with a pH of 7.8, elongate 5 to 10 times the length of the original bacillus isolated on blood agar medium as soon as they begin to grow luxuriantly. This occurs approximately 10 transfer generations removed from the parent strain, at which time, the organisms become relatively less virulent and less antigenic.

When freshly isolated ovoid shaped bacilli are transferred from blood to chocolate brown agar medium, the organisms elongate 2 to 10 times the length of the original, when only 20 plus generations removed from the parent strain. When they are only 10 generations removed, the organisms grown on chocolate brown agar medium possess but one-fifth of their former virulence.

When grown in our special veal brain broth the organism becomes branching and filamentous, resembling mycelium, whose size is difficult to measure. When transferred back to veal brain agar, the mycelium-like organisms immediately assume their bacillary forms.

When long bacillary appearing organisms, acclimated to veal brain

agar, pH 7.8, are re-acclimated (140 generations of re-transplanting) to hemoglobin blood agar medium having a gradual decreasing pH, they become small in size and length.

M19. Experimental Rabies in White Mice. Studies on Passive Immunization II. ANSON HOYT, ROY T. FISK, FREDERICK J. MOORE AND RALPH L. TRACY, School of Medicine, University of Southern California, Los Angeles.

Experiments were performed to evaluate the efficacy of anti-rabic serums, using white mice as test animals. Fixed virus (Cutter strain recently passed through rabbits) was employed. This virus, as prepared, produced rabies in 153 out of 154 control mice upon intracerebral inoculation of constant amounts of a 1/400 dilution but the inoculation of a 1/6400 dilution brought down only 36 out of 80 mice. The serums (prepared by hyperimmunization of rabbits and goats) neutralized equal volumes of 1/200 virus *in vitro* to a titer of over 1/1000.

A summary of tests, in which large intraperitoneal anti-serum injections were made shortly before intracerebral virus inoculation, shows the following results: (*a*) A higher percentage of the 188 treated mice (36 per cent) survived than of the 247 corresponding controls (9 per cent). (*b*) A longer mean incubation period was shown by each group of treated mice that contracted rabies than by the corresponding controls. Anti-serum injected either 16 days before or shortly after virus failed to protect. Treatment with normal serums or with anti-normal rabbit brain serum was ineffective.

In further experiments anti-serum injected intracerebrally one-half hour before intracerebral virus inoculation afforded excellent protection, but anti-serum injected either 24 hours before or 2 hours after virus was ineffective.

Anti-serum, injected intraperitoneally either 4 days or a few hours before intramuscular virus inoculation, gave excellent protection and a slight protection was also indicated by the administration of anti-serum one day after virus.

M20. Purification of Suspensions of the Virus of Rabies. C. A. BEHRENS AND J. F. BARKER, Purdue University.

The vaccines used to immunize against rabies contain the virus and a great deal of nervous tissue. Our purpose was to prepare suspensions which contained as much of the virus and as little of the tissue as possible.

The method employed is based upon precipitating the tissue at its iso-electric point by the use of M/100 citric acid. It was determined that the iso-electric point of brain tissue was between pH 4.8 and 5.0.

Water clear preparations of the virus have been prepared from which as high as 65 per cent of the protein has been removed.

M21. Concerning the Relationship of the Virus Inducing Rabbit Papillomas to the Cancers Developing Therefrom. PEYTON ROUS, J. W. BEARD AND JOHN G. KIDD, The Rockefeller Institute for Medical Research.

As already reported, cancers have arisen in papillomas induced in domestic rabbits with the virus responsible for the "natural" growths of cottontails. The strains of virus employed are not recoverable in active form, so our study of their relationship to the cancers has required indirect methods.

Extensive observations confirm the finding that only those papillomas become cancerous which are engendered by a notably active virus. Additional proof has been obtained that the papilloma itself has malignant potentialities at an early stage, that the cells affected by the virus are those which become cancerous, and that the formative influence of the virus is perceptible in many of the ultimate tumors. The blood of rabbits in which one of the latter, a squamous cell carcinoma, had grown after transplantation, neutralized the virus *in vitro* whereas that of controls and of rabbits unsuccessfully implanted with the same tumor material proved devoid of such power. A possible transfer to the new hosts of papilloma cells in the cancerous material had been excluded by utilizing a metastatic nodule for transplantation purposes.

The observed differences in the individual cancers can be readily explained by variant changes in a virus stimulating the cells; and certainly a virus causes and accompanies the papilloma, gives it neoplastic characters, acts to determine carcinosis, persists in the cancers and influences their character. Nevertheless a hypothetical, superimposed cause can still be invoked as responsible for the malignancy.

M22. The Cultivation of the Virus of Lymphocytic Chorio-meningitis in the Developing Chick Embryo. IDA A. BENGTSON, National Institute of Health, Washington, D. C.

The virus of lymphocytic chorio-meningitis was successfully cultivated through 8 successive passages in the chorio-allantoic membrane

and in the brain of the developing chick embryo in accordance with the technique of Goodpasture.

A computation of the total dilution of the final passage indicates that the virus was present in a dilution of at least 1×10^{-21} of the original virus.

Dilutions of 1×10^{-2} and 1×10^{-3} of the membrane or brain of one generation were usually infective for the succeeding generation.

The virus at each passage was tested on mice by intra-cerebral inoculation. The typical symptoms of chorio-meningitis developed each time using dilutions of 1×10^{-2} and 1×10^{-3} and occasionally 1×10^{-4} of the embryo membrane and brain.

The virus was present in the liver as well as in the membrane and brain of the embryo and it was also present in the amniotic fluid.

The chick embryos survived longer than the mice, indicating a higher virulence of the virus for the mouse than for the chick embryo. The lesion on the membrane of the embryo at the site of inoculation was slight, though in some cases there were present small, discrete, irregular, cloudy areas below the surface of the ectodermal layer.

M23. Relationship of Sarcoma to Leukosis of Chickens. J. FURTH, Department of Pathology, Cornell University Medical College, New York, N. Y.

Recent findings lead to the assumption (Oberling and Guérin) that oncogenic viruses may readily adapt themselves to different types of cells which they stimulate to unrestricted growth.

We have studied two complex strains in addition to simple strains of leukosis and sarcoma and their artificial mixtures.

The complex Strain 12 yields bone sarcoma, lymphomatosis and a combination of the two diseases. Transmission with blood of chickens free from bone tumor yields leukosis only, whereas transmission with blood of tumor bearing chickens yields both bone tumor and leukosis.

Attempts at separation of a leukosis virus from the complex Strain 13, which yields sarcoma (endothelioma) and erythroleukosis, have been unsuccessful. Pure cultures of this sarcoma grown *in vitro* for 67 days yielded erythroleukosis and sarcoma. The virus of the erythroleukosis Strain 1 dies within approximately two weeks in *in vitro* cultures of fibroblasts or common chicken sarcoma cells.

When viruses of a pure sarcoma (No. 11) and a pure erythroleukosis (No. 1) are mixed, they do not help each other, but retain their identity and can be readily separated by various procedures. E.g. (*a*) Tissue

cultures of the sarcoma cells derived from chickens that have both sarcoma and leukosis yield the pure sarcoma virus; (*b*) intravenous transmission with small amounts of blood, on the contrary, is likely to yield the leukosis virus only.

Thus one complex strain (No. 13) appears to be produced by one virus, the other (No. 12) by two viruses. The assumed adaptability of oncogenic viruses to different types of cells requires stronger evidence than has hitherto been presented.

M24. Experiences in Several Epidemics with Experimental Measles in Monkeys. FRANCIS G. BLAKE, JAMES D. TRASK, CHARLES S. CULOTTA AND AGNES R. BEEBE, School of Medicine, Yale University.

Experimental measles was produced in monkeys (*Macacus rhesus*) by the intratracheal inoculation of nasopharyngeal washings collected from patients in the early stages of measles. Material was obtained in epidemics of 1920, 1922, 1932 and 1934. In each year experimental measles was obtained. Serial passage of the disease from monkey to monkey was successfully performed by a variety of methods, including mere contact. However, in all cases, after 2 to 12 generations, passage failed; thus in unlimited series successful passage was not obtained by any method.

M25. A Fatal Virus Disease in Ferrets. C. A. SLANETZ, H. SMETANA AND A. R. DOCHEZ, Columbia University, College of Physicians and Surgeons.

In October, 1934, a disease appeared spontaneously in a group of experimental ferrets with a mortality of 100 per cent. Ferret breeders reported a similar disease.

Sixty-five ferrets, 5 puppies and a number of mice were used in this study. Normal ferrets readily became infected by contact with sick individuals, or by intranasal instillation, under light ether anaesthesia, of filtrate from a 10 per cent lung tissue emulsion. The disease in ferrets produced by intranasal inoculation had a relatively constant incubation period and uniform clinical symptoms. Four to 5 days following inoculation with a bacteriologically free, Berkefeld N filtrate of lung tissue an elevation in temperature, 104–106°F., occurred which generally persisted until the animal died. On the sixth or seventh day one or both eyes had a tendency to close. Finally both eyes were found closed with a concomitant loss of appetite. The average survival

period following artificial infection was 25 days. Cultures from the lungs and liver yielded a hemolytic streptococcus.

At autopsy the gross pathological changes of the viscera were rather slight and consisted mainly of congestion and hemorrhages of the lungs, fatty changes in the liver and enlargement of the spleen. Areas of bronchopneumonia were usually due to secondary infection.

Microscopically there were patches of hemorrhagic pneumonia, acute splenic tumor, marked fatty changes in the liver, with occasional focal necroses. Round, homogeneous cytoplasmic inclusion bodies were found in the lining cells of bronchi, kidney pelves and urinary bladder.

Immunization of ferrets was effected by injecting subcutaneously 2 cc. of a formolized lung tissue suspension at bi-weekly intervals. After 3 injections the vaccinated ferrets proved insusceptible to living ferret virus.

Ten cc. of virus containing filtrate injected into 5 puppies, approximately 3 months of age elicited no temperature response. The same puppies proved susceptible to canine distemper virus when injected 4 weeks later.

No relationship was established between human influenza virus and the ferret virus.

M26. Immunological and Serological Evidences of a Close Relationship between the Viruses of Rabbit Fibroma (Shope) and Infectious Myxomatosis (Sanarelli). GEORGE P. BERRY AND JOHN A. LICHTY, JR., University of Rochester, School of Medicine and Dentistry.

Shope reported in 1932 that the viruses of rabbit fibroma and infectious myxomatosis were immunologically related. Shope's findings have been confirmed and extended.

Of 60 fibroma-recovered domestic rabbits, inoculated with myxoma, 70 per cent survived. Using their sera a technique was developed capable of detecting in serum minimal neutralizing capacities against either virus. Neutralization was tested in the skin of the domestic rabbit with amounts of virus which were low multiples of the minimal skin taking dose. The tests were adequately controlled.

Sera from 6 fibroma-convalescent domestic rabbits repeatedly neutralized myxoma virus as well as fibroma virus, frequently in high titre. This finding, contrary to Shope's resulted we believe from the use of a more sensitive technique than he employed.

Infectious myxomatosis has been established in cottontail rabbits

(genus, *Sylvilagus*) and carried to date through 13 serial transfers. This is the first time the disease has been established in any host other than the domestic rabbit (genus, *Oryctolagus*). Without failure, 35 cottontails developed myxomatosis after intracutaneous or intravenous inoculation. None died. Fifteen subsequently failed to "take" after inoculation with fibroma virus. Fifty other cottontails were injected with fibroma virus. Subsequently those tested were resistant to both viruses. Moreover, sera from myxoma-convalescent cottontails neutralized fibroma virus as well as myxoma virus.

Thus, infection with the viruses of rabbit fibroma and infectious myxomatosis induces a well-marked cross-immunity. The resistant state following either infection is accompanied by reciprocal neutralizing properties of the sera.

M27. A Method for Changing the Virus of Rabbit Fibroma (Shope) into that of Infectious Myxomatosis (Sanarelli). GEORGE P. BERRY AND HELEN M. DEDRICK, University of Rochester, School of Medicine and Dentistry.

The close immunological relationship between rabbit fibroma and infectious myxomatosis led us to explore the hypothesis that the viruses in question are but different strains of one basic virus. Our approach was suggested by Griffith's studies in 1928 on the transformation of pneumococcal types. We believe that we have changed the virus of rabbit fibroma into that of infectious myxomatosis.

The method consisted in inoculating domestic rabbits with a mixture of active fibroma virus and heat inactivated myxoma virus. The fibroma virus was a suspension of pooled dermal and testicular fibromata, the myxoma virus a suspension of primary skin lesions. The myxoma suspensions were inactivated for 30 minutes at 60°C., at 75°C. and at 90°C. Transformation from fibroma to myxoma virus occurred with "60°C. material" in 6 experiments, with "75°C. material" in 2, but not with "90°C. material." Test rabbits developed characteristic fatal myxomatosis, with typical histopathology. Secondary lesions yielded myxoma virus on transfer, and normal animals were infected by simple contact.

Control rabbits outnumbered test animals. Inoculations and serial transfers of heat inactivated myxoma suspensions were negative, as were the results of mixing such suspensions with a variety of viruses and other materials.

These experiments suggest that something in myxomatous material,

not destroyed at 75°C., lends virulence to fibroma virus, possibly in a manner analogous to bacterial haptenes. This approach may well prove useful in exploring other virus relationships, for instance in the animal pox group, in the foot and mouth disease group, etc.

M28. Etiology of Trachoma. PHILLIPS THYGESON, University of Iowa, Iowa City.

In studies of the bacterial flora in trachoma, no bacterium was demonstrated in the lesions of trachoma, by bacterioscopic or cultural methods, in sufficient frequency to indicate etiologic significance. The various bacteria isolated proved incapable of producing the disease when inoculated in either pure or pooled cultures on the human conjunctiva.

Preliminary filtration experiments with Berkefeld N and Chamberland L2 and L3 filters were negative. In view of successful filtrations reported by Nicolle, Cuénod, and Blaizot, who used a specially constructed filter of small filtration area, further experiments were made with Elford graded collodion membranes in which adsorption losses are minimal. From pooled conjunctival scrapings from selected cases of trachoma in Indian children, infective bacteria-free filtrates were obtained in four experiments on baboons with use of 0.75 micron A.P.D. membranes, and in one experiment on the human conjunctiva with use of 0.6 micron membranes. The conclusions of Nicolle, Cuénod and Blaizot as to the virus etiology of trachoma were thereby confirmed.

The possible identity of the elementary body (Halberstaedter Prowazek) with trachoma virus was examined. The virus nature of these bodies was considered probable from their morphologic similarity to the elementary bodies of other virus diseases, particularly to those of inclusion conjunctivitis and psittacosis. That they may have an etiologic rôle in trachoma is suggested by (1) their absence in conjunctivitis of bacterial origin; (2) their presence in the one infective filtrate examined; (3) their demonstration in four cases of trachoma followed from onset; (4) their presence in large numbers in three cases of pure trachoma with acute or sub-acute symptoms; and (5) their demonstration in three series of active trachoma cases in percentages of 40, 44, and 90. Opposing the conception of a primary rôle for the elementary bodies stand (1) the difficulty, and sometimes impossibility, of demonstrating them in certain cases; and (2) the failure up to the present time to demonstrate them in experimental trachoma of the *Macacus rhesus* monkey. No definite conclusions are justifiable at the present time.

M29. Inactivation of the Virus of Typical Tobacco Mosaic and of Escherichia coli in the Shorter Ultra-violet. B. M. DUGGAR AND ALEXANDER HOLLAENDER, Laboratory of Plant Physiology, University of Wisconsin, Madison.

A highly purified, stirred suspension of the virus of typical tobacco mosaic was irradiated in the presence of *Escherichia coli* with measured quantities of monochromatic ultra-violet radiation from λ 2250 to 3000 Å. The irradiation was performed so that it was possible to compare the relative resistance as well as the wave-length dependence of inactivation of the virus and bacteria.

It has been found that the amount of energy necessary to destroy 50 per cent of the virus in suspension (physiological salt solution) at λ 2250 Å is only one-fifth of the amount required at 2650 Å. Whereas the energy necessary to inactivate bacteria in the same suspension is higher at λ 2250 than at 2650 Å.

M30. Inheritance of Susceptibility to Mosaic Disease in Pepper Plants. F. O. HOLMES, The Rockefeller Institute for Medical Research, Princeton, N. J.

There are three known types of response to infection with tobacco-mosaic virus among varieties of the garden pepper, *Capsicum frutescens*. These are localized necrosis, systemic necrosis, and persistent mottling. The three types of response are heritable. In hybrids the first is completely dominant and the second imperfectly dominant with respect to the third.

M31. The Isolation of a Crystalline Protein Possessing the Properties of Aucuba-Mosaic Virus. W. M. STANLEY, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

A crystalline protein, which has the properties of the virus of aucuba mosaic, has been isolated from extracts of Turkish tobacco plants affected by aucuba-mosaic disease. The methods employed in the concentration, purification, and crystallization of the protein were similar to those previously used for the isolation of crystalline tobacco-mosaic virus protein. The crystals obtained from extracts of plants having aucuba mosaic are needles similar to the crystals of tobacco-mosaic virus protein. The nitrogen content and infectivity of crystalline aucuba-mosaic virus protein were unchanged after 3 successive crystallizations. The material is between 100 and 1000 times more infectious than ordinary juice preparations. A solution of the crystals

gives a precipitate when mixed with sera of animals injected with a solution of crystalline tobacco-mosaic virus protein. When solutions are made more alkaline than about pH 11 or more acid than about pH 1, or are heated to about 94°C., the protein is denatured and its activity lost, as was found to be the case with tobacco-mosaic virus protein. These points of similarity are not surprising, since aucuba mosaic is regarded as a severe strain of the tobacco-mosaic disease. Although aucuba-mosaic virus protein crystals and tobacco-mosaic virus protein crystals are strikingly similar in appearance and in their physical and chemical properties, their biological properties are different, for, on inoculation to susceptible plants, the one gives rise to aucuba mosaic and the other to tobacco mosaic.

M32. Studies on Plant Viruses: Serological Reactions and Plant Immunity. JORGEN M. BIRKELAND, Department of Bacteriology, Ohio State University.

A comparison of the results obtained by the serological reactions of plant viruses and induced or acquired plant immunity reactions shows: (1) that viruses which induce complete cross-immunity in plants are serologically indistinguishable, (2) that viruses which induce partial cross-immunity in plants show a serologic relationship, and (3) that viruses which do not induce cross-immunity in plants are serologically unrelated.

M33. A Study of Inactivated Yellow Fever Virus as an Immunizing Agent. JOHN E. GORDON AND T. P. HUGHES, Rockefeller Foundation.

Yellow fever virus inactivated by heat, by exposure to ultra-violet light or by formaldehyde did not possess any demonstrable immunizing property when tested in rhesus monkeys, regardless of variations in dosage, in the duration of the course of immunization or in the route of injection. There was no evidence that the virus became, at any stage in inactivation, non-infective but still antigenic. In these experiments immunity occurred only as the result of a demonstrated infection.

M34. A High Speed Centrifuge for Study of Viruses. J. H. BAUER AND E. G. PICKELS, Rockefeller Foundation.

The centrifuge, built on the principles described by Beams and Pickels (Rev. Sc. Instr., 1935, **6**: 299) is driven by compressed air.

Both the stator and the rotor measure 8 inches in diameter. The stator is made of steel and has 24 air jets, each $\frac{1}{16}$ inch in diameter. It is mounted on a rubber cushion and, to insure flexibility, is supported on the sides by springs, the tension of which can be regulated. The rotor is machined from a solid block of duraluminum and weighs 21.5 pounds. It has 48 flutings, each 3 inches long and $\frac{1}{8}$ inch deep. The total height of the rotor is 7 inches. Eight holes, each measuring one-half inch in diameter and 3 inches deep, are bored in the top portion an equal distance apart and at an angle of 40° from the axis of rotation. Containers of cellulose composition (furnished by the Lusteroid Container Company, South Orange, N. J.) fit into these holes. Each container has a capacity of 7 cc. and is transparent and flexible. It weighs 80 per cent less than glass and withstands high centrifugal force. The material to be centrifuged must be covered with a layer of oil to prevent evaporation. The highest speed obtained was 16,000 r.p.m., which corresponds to a centrifugal force of 20,000 times gravity in the central portion of the containers. An air pressure of nearly 200 pounds per square inch is required to attain maximum speed. The rotor has no bearings and rotates on a layer of compressed air which cools slightly on expanding and therefore can be run for long periods without danger of heating.

M35. The Kinetics of Bacteriophagy. CHARLES E. CLIFTON, Stanford University, California.

The time required for an anticoli phage to lyse an actively growing culture of *Escherichia coli* to an arbitrary turbidity end-point, as measured with the aid of a photoelectric turbidimeter, is, under uniform cultural conditions, proportional to the initial concentration of phage. This time factor furnishes a basis for the quantitative estimation of phage.

Both bacterial multiplication and increases in phage concentration, in gently stirred Martin's broth cultures at 32°C., are preceded by short lag periods, during which 90 to 95 per cent of the phage is taken up by the bacteria. During the period of logarithmic bacterial growth, the rate of phage production is also logarithmic but proceeds more rapidly. During this period the ratio of extracellular phage to total phage has a small but relatively constant value. Lysis is initiated at the time the ratio of phage to bacteria exceeds a certain critical value. Under the conditions of these tests this ratio was 1600 phage units to a bacterium. The lytic destruction of the cells is an extremely rapid process,

the rate of lysis being logarithmic and apparently independent of the concentration of phage.

The time required for lysis to reduce the concentration of bacteria, in an actively growing culture of *Escherichia coli*, to an arbitrary turbidity end-point is increased in the presence of a specific antilytic serum. Quantitative measurements, under the dynamic conditions of these tests, indicate that the neutralization of phage goes almost to completion within a few minutes. This applies over a wide range of concentrations of phage and antiserum. The final equilibrium may, however, not be established for several hours or days. The bearing of these results on the general problem of immunity in virus infections is discussed.

M36. Inhibition of Staphylococcus Bacteriophage and the Virus of Vesicular Stomatitis. MORRIS L. RAKIETEN, TONY L. RAKIETEN AND SIMON DOFF, Department of Immunology, Yale University School of Medicine and the Department of Bacteriology, Long Island College of Medicine.

Susceptible cultures of staphylococci heated at 60° or 120°C. are able to adsorb staphylococcus bacteriophages within 18 hours at incubator temperature. Phage resistant staphylococci, on the whole, are devoid of action on these same phages. Extracts of susceptible and resistant cultures of staphylococci prepared according to the method of Burnet (Jour. Path. and Bact., 1934, **38**: 385) show the same inhibitory effect as heat killed cultures. There appears to be a relationship between susceptibility of a culture to a phage and the potency of the extract from the same culture. However, one does encounter phage resistant strains of staphylococci which in their viable state are able to completely adsorb phage; but extracts made from these cultures have no demonstrable inhibitory effect on "staphy" phages.

Several strains of bacteria belonging to the subtilis group have been isolated which are also able to completely adsorb "staphy" phages. These organisms have no inhibitory effect on phages that attack members of the enteric group of bacteria, nor on streptococcus bacteriophages. The adsorption of phage to these bacilli takes place within 18 hours at 35°C. Once the phage has been inactivated by the presence of the non-susceptible bacilli, neither the addition of susceptible staphylococci, nor lysis of the subtilis strain to which the phage has been adsorbed frees any of the staphylococcus bacteriophage. Spores of these subtilis strains lack the ability to adsorb phage. A serum produced against one of these strains of subtilis agglutinates in high dilution

several of our strains of staphylococci suggesting that a portion of the antigenic complex of the subtilis is closely related to staphylococcus antigen.

One of these strains of subtilis has also been studied with respect to its inactivating power on a virus other than phage. The culture injected intracerebrally into mice is non-pathogenic. A 1:100 suspension of a virulent race of vesicular stomatitis virus is rendered impotent after being in contact with this culture of *Bacillus subtilis* for 15 hours at 35°C. Apparently, the same relationship between cell growth of this culture of subtilis and adsorption of staphylococcus phages also holds true with the virus of vesicular stomatitis.

M37. A Bacteriophage for the V Form of Salmonella typhi. JAMES CRAIGIE, Connaught Laboratories, University of Toronto.

A bacteriophage which appears to be specific for the V form of *Salmonella typhi* (*Bacillus typhosus*) was obtained from a culture isolated from the faeces of a case of typhoid fever. Cultures of the pure V form of the strains Rawlings (Bensted), Watson, Ty 2 and others are sensitive to this bacteriophage while Ty 8, Ty 901 (H and O) and other W strains are resistant. All other *Salmonella* species (antigenically representative of the genus) which have been tested are resistant. Secondary growth obtained after lysis of the V form are serologically W in type and of lowered virulence for the mouse. No resistant V strains have been encountered.

In collaboration with Dr. K. F. Brandon, over 200 strains of *Salmonella typhi* from recent cases and carriers have been examined serologically. The majority of these were found to be intermediate V forms of such transient resistance to O agglutination in each subculture as to necessitate the individual examination of a number of young, well isolated, actively developing colonies. Examination of these cultures for sensitivity to the bacteriophage (by addition of a drop of bacteriophage filtrate to a freshly inoculated plate culture) has yielded results consistent with the previous serological findings. (Note: The terminology (V form and W form) introduced by F. Kauffmann (Z. Hyg. **116:** 617) is employed in this abstract without endorsement of his hypothesis of V-W variation.)

M38. Bacteriophage and Antivirus Therapy of Localized Experimental Infections with Staphylococcus. J. BRONFENBRENNER AND S. E. SULKIN, Department of Bacteriology, Washington University Medical School, St. Louis, Missouri.

In spite of numerous reports of clinical improvement following application of bacteriophage therapy in infections due to staphylococcus, its usefulness in experimental infections has not been sufficiently studied. In a preliminary investigation of the therapeutic value of bacteriophage in experimental staphylococcus skin infections in rabbits, no therapeutic effect was obtained. On the contrary, certain bacteriophage filtrates, instead of promoting the healing have caused a temporary intensification of the local reaction.

Suspecting that this effect might have been due to the presence of the "Reynals spreading factor" in some of the bacteriophage lysates, several groups of animals were infected by injection of different strains of staphylococcus intracutaneously, intracorneally, or into the anterior chamber of the eye and treated by application of bacteriophage propagated on organisms of different degrees of invasiveness. It was found that the bacteriophage propagated upon the highly invasive strain of staphylococcus invariably produced a temporary exacerbation of the lesions. That these lysates contain "spreading factor" is suggested by the fact that bacteriophage propagated upon the highly invasive staphylococcus produced similar intensification of the lesions after having been inactivated by heat.

In a parallel series of experiments, rabbits and guinea pigs injected intracorneally or into the anterior chamber were treated by local application of Besredka's antivirus. In these experiments it was likewise found that the course of infection was not influenced when antivirus obtained with non-invasive strains of staphylococcus was applied, while antivirus prepared with highly invasive strains produced a temporary enhancement of the lesions analogous to that produced by application of bacteriophage propagated on invasive strains of staphylococci.

From these data it appears that in localized staphylococcus infections in animals no beneficial results are obtained by the application of bacteriophage or antivirus. Moreover, unless care is taken to avoid use of invasive strains of staphylococcus in their preparation, the application of bacteriophage or antivirus to the lesions may result in undesirable intensification of the local reaction.

M39. Comparison of Animal Inoculation Tests with in Vitro Reactions of Staphylococci and Streptococci. GEORGE H. CHAPMAN, Clinical Research Laboratory, New York, N. Y.

As ordinarily made, animal inoculation tests which depend upon the

lethal effect of staphylococci and streptococci were found to be inaccurate. More reproducible results were obtained by: (1) titrating each culture with a number of animals (particularly because of varying resistance), (2) injecting an accurately calculated dose, and (3) establishing arbitrary time limits (4 to 48 hours for mice and from 4 hours to 6 days for rabbits). Because they may have been due to shock, emboli, intercurrent infection, etc., irregular deaths which occurred before or after the arbitrary time limits were ignored. Under these experimental conditions, the reproducibility, as judged from the injection of sub-lethal doses into 434 animals, was from 84.5 to 100 per cent, depending on the type of experiment.

The results with *in vitro* tests were as follows: The violet agar reaction and hemolysis and coagulase tests agreed in 96.5 per cent of 510 strains of *Staphylococcus albus* and in 90.2 per cent of 305 strains of *Staphylococcus aureus*. Agreement in the resistance of streptococci to hexylresorcinol and sodium bicarbonate occurred in 97.0 per cent of 186, 98.9 per cent of 387, 99.42 per cent of 260, and 99.46 per cent of 279 strains, respectively. The improved correlation of the later experiments was attributed to increased technical skill. The correlation of the *in vitro* tests with animal inoculation tests was about 95 per cent. The *in vitro* tests were simpler; they could be completed in a shorter time and were less expensive.

M40. Direct Bacteriological Experimentation on the Living Mammalian Fetus. ORAM C. WOOLPERT, Department of Bacteriology, Ohio State University.

The adaptability of the fetus to bacteriological experimentation and the comparative susceptibility of fetal animals to certain disease agents were studied. Successful inoculations were made principally in guinea pigs, of fetal ages from 25 to 65 days. The youngest fetuses were inoculated intraplacentally and those of intermediate age intracerebrally, by needle puncture through the maternal uterine wall following maternal laparotomy under anesthesia. Older fetuses were inoculated without operative exposure by needle puncture through the maternal abdominal wall. Experiments were terminated usually by cesarean section. The most favorable fetal age for inoculation was found to be 40 days or younger in guinea pigs. Fetal guinea pigs were inoculated with poliomyelitis virus, vaccinia virus, the submaxillary gland virus of guinea pigs, *Mycobacterium tuberculosis* (H37 and BCG), and diphtheria toxin. Each of these agents, except the poliomyelitis virus,

produced characteristic disease processes and death, when administered in adequate amount. The fetus proved more susceptible than the postnatal animal in the case of vaccinia, the submaxillary virus and BCG.

It is believed that these experiments establish the practicability of the technic and suggest a wide field of usefulness for the fetus in bacteriological studies because of its freedom from external contamination and greater susceptibility to certain infectious agents.

M41. Susceptibility of the Fetal Guinea Pig to the H37 Tubercle Bacillus and to the Bacillus of Calmette-Guerin. I. S. NEIMAN AND ORAM C. WOOLPERT, Department of Hygiene and Bacteriology, University of Chicago, and Department of Bacteriology, Ohio State University.

Utilizing a technic described by Woolpert, a study was made of the comparative reactions of the fetal guinea pig to infection by the H37 tubercle bacillus and Bacillus Calmette-Guerin (BCG). Inoculations were made principally intracerebrally and the experiments were terminated either by cesarean section or spontaneous delivery. Gross, histological and cultural examinations showed that both H37 and BCG were capable of producing characteristic progressive tuberculosis in the fetal guinea pig, but H37 was the more pathogenic and disseminated more widely. Local reactions noted were caseation and liquefaction of brain tissue, and meningitis. Tubercle formation in liver, spleen and lungs was common with the larger dosages. Although numerous acid fast bacteria were demonstrated in smears and sections of involved tissues, cultural recovery of BCG was irregular and fetus-to-fetus transfer was not accomplished in the few attempts made. The feasibility of the technic for fetal experimentation is confirmed and the usefulness of the fetal guinea pig for studies in tuberculosis is indicated.

M42. Susceptibility of the Guinea Pig Fetus to Vaccinia. JOSEPH STRITAR AND N. PAUL HUDSON, Department of Hygiene and Bacteriology, University of Chicago, and Department of Bacteriology, Ohio State University.

Woolpert has evolved a technic for mammalian fetal inoculation and found the guinea pig to be peculiarly susceptible to certain infectious agents. We have investigated the reaction of the guinea pig fetus to vaccinia, the Levaditi strain being employed.

Gross pathologic changes were observed in various tissues of 88 out of 115 fetuses inoculated *in utero*; most injections were intracerebral.

The parts found involved a few days after inoculation were the skin, brain surfaces, lungs, liver, kidney and spleen, predominantly the lungs and kidneys. The skin lesions had the appearance of typical pox. The essential microscopic pathology was necrosis, edema and hemorrhage.

The vaccine virus was regularly recovered from fetal parts, most frequently and in highest titer from the kidney. It was passed in a series of fetuses without apparent alteration in respect to the fetus or rabbit skin.

To summarize, vaccinia virus that caused in the postnatal animal only the slightest reaction, and that rarely was propagated in the guinea pig fetus with generalized infection and local lesions resulting. It is proposed that this peculiar susceptibility of the fetus may be due to its general immaturity and rapid cell growth.

M43. The Relative Susceptibility of Fetal and Newborn Guinea Pigs to the Submaxillary Gland Virus. FLOYD S. MARKHAM AND N. PAUL HUDSON, Department of Bacteriology, Ohio State University.

Experimental work with the guinea pig submaxillary gland virus is restricted by the fact that the natural host is the only susceptible animal available and that it may become spontaneously infected within the first few days of life. Woolpert's development of a practical technique of fetal inoculation opened a new avenue of approach to the problems of tissue susceptibility and immunity to the submaxillary gland virus.

The present work involved 60 guinea pig fetuses studied at intervals of 3 to 21 days after intracerebral inoculation. Dilutions of virus which produced no more than a slight and transient temperature rise in intracerebrally inoculated guinea pigs less than two weeks old, induced in the fetus extensive and characteristic lesions in the brain, liver and placenta.

It was found that the immune state of the mother, dependent on the presence or absence of spontaneous infection, did not influence the susceptibility of the fetus. Similarly, young guinea pigs inoculated intracerebrally succumbed to the virus irrespective of the immune state of the mothers they suckled.

Therefore, it appears that the marked susceptibility of the guinea pig fetus makes it the animal of choice for detection of virus and for securing tissues rich in virus, and that humoral antibodies are not conferred in effective amounts either *in utero* or in the milk.

M44. A Quick and Reliable Method for Staining Smears for Gonococci in a Public Health Laboratory. SETH T. WALTON, City Health Service, Charlotte, North Carolina.

A modified Pappenheim's stain, using methyl green and pyronin in carbolized water with glycerine is used for the quick detection of *Neisseria gonorrheae* in smears. Gonococci stain bright red while other Gram-negative and Gram-positive organisms are purple, or reddish purple. Nuclei of cells are green and the cytoplasm is a delicate shade of rose. With such a contrast of colors it is much easier to detect gonococci when present in small numbers, and especially in vaginal smears, than when all the cellular material is stained the same color. When the slide is warmed slightly in the flame just before staining, one-half minute is sufficient and longer staining is to be avoided.

M45. The India Ink Reaction. LASZLO DETRE, National Institute of Health, Washington, D. C.

Early work of Dineur, C. Nicolle, Kraus, Dean and Arkwright connected the agglutination with precipitation of bacillary extracts by homologous antiserums. In 1933, the author demonstrated under the title, "The India Ink Reaction," a phenomenon which utilizes the surface activities of a colloidal suspension to make inapparent or subvisible antigen-antibody reactions detectable. It was shown that a saline-extract of *Bacillus typhosus* and other bacteria, when mixed with their antiserums—together with a small amount of the ordinary India ink, gave a speedy flocculation and subsequent decolorization. Under optimal conditions the reaction is clearly manifest within one minute and may reach its maximal intensity within a half hour.

Subsequent studies disclosed the following forms of India ink reaction:

1. Floccular reactions, characterized by speedy formation of dark brown or black floccular precipitates, and strong decolorization.
2. Granular reactions, characterized by the late appearance of uniform small dark granules and slight or absent decolorization effect. Their intensity depends: (*a*) on individual properties of the examined species or strains, (*b*) on the culture media, growth temperature and method of extraction, (*c*) on salt content, pH, and the presence or absence of protective colloids in the medium, (*d*) on temperature and time of the reaction, and (*e*) on the ratio of antigens and antibodies.
3. Normal fresh serums may precipitate India ink in two ranges:

(*a*) in concentrated (1/5–1/10) serums, using 0.1 per cent saline as diluent, (*b*) in a zone from about 1:200–1:1000.

This "normal zonal reaction" is a manifestation of a hitherto undescribed form of fibrinogen-thrombin union. It can be lessened or eliminated in various ways.

4. The use of the ink reactions is advocated for qualitative antigenic and antibody analysis.

M46. The Relationship of Antianaphylaxis to Antibody-Balance and to Immunity. MARION C. MORRIS AND J. BRONFENBRENNER, Washington University School of Medicine, St. Louis.

That antianaphylaxis is not due to an excess of circulating antibody was shown by injecting additional antibody into the circulation of passively sensitized guinea pigs shortly before injection of antigen. Such animals were not protected against anaphylactic shock to any greater extent than sensitized guinea pigs injected intravenously with normal serum before testing with antigen. It required smaller amounts of antigen to kill guinea pigs which had received additional antibody than in the case of guinea pigs which had not received additional antibody.

Similarly, antianaphylaxis cannot be explained on the basis of antibody depletion, since desensitized guinea pigs when resensitized fail to attain the same degree of reactivity as normal animals given the same amount of antibody. Furthermore, when mouse protection tests were used to determine directly the content of circulating antibody in guinea pigs: (*1*) passively sensitized with anti-Friedlander type B antiserum; and (2) similarly sensitized and subsequently desensitized with the specific carbohydrate, it was found that desensitization has no appreciable effect upon the content of circulating antibody. Resistance of sensitized guinea pigs to infection with virulent Friedlander's bacillus, being dependent upon the same antibodies, was likewise shown to be unchanged by desensitization.

It was concluded that anaphylaxis and immunity are different manifestations of the same antigen-antibody reaction and that a state of antianaphylaxis is due to non-specific changes and independent of antibody-balance.

M47. Hypersensitivity to Certain Bacterial Extracts, as Demonstrated by Changes in the Nonfilament-Filament Ratio of Neutrophilic Leucocytes. MERRITT H. STILES, 113 W. Chestnut Hill Ave., Philadelphia.

Effects noted in persons with chronic infections after the injection of certain autogenous bacterial preparations suggested the presence of extreme hypersensitivity to the extracts used. In view of published reports of the relationship between disease activity and variations in the "Schilling" index, hematological studies were made to determine whether leucocytic changes occurred after the administration of extremely weak dilutions of these extracts.

The bacteria used were either: (*a*) streptococci which were resistant to sodium bicarbonate and hexylresorcinol, (*b*) hemolytic strains of *Staphylococcus aureus*, (*c*) staphylococci which coagulated oxalated plasma, or (*d*) a combination of these organisms. Suspensions containing one billion bacteria per cubic centimeter in one per cent phenol were diluted to from 10^{-30} to 10^{-40} and injected either subcutaneously or intravenously into the persons from whom the strains had been obtained.

After injection of certain "doses" of these extremely dilute extracts definite changes occurred in the nonfilament-filament ratio of neutrophilic leucocytes, the particular type of change in each instance depending upon the relative quantity injected. The hematologic changes usually paralleled changes in the clinical condition. The injection of diluting fluid alone did not produce any comparable effects.

The individuals tested were still further hypersensitized for variable periods after each injection (even after favorable hematologic and clinical response). Frequently violent reactions occurred if the injection was repeated during these periods. However, if the secondary injection was delayed until *after* the period of increased sensitivity, the original response was reproduced.

M48. The Bacteriology and Bacteriallergy in Chronic Infections. ABRAHAM S. GORDON, Brooklyn Jewish Hospital.

Most chronic infections which present clinical problems are low grade but active infections to a greater or lesser degree. Specifically rheumatoid arthritis has been studied. This chronic disease is assumed to be of infectious etiology and is due in most cases to one or another variety of streptococcus. The author believes that chronic streptococcic disease is a definite clinical entity and is a result of infections and reinfections with the same or similar strains of bacteria which gradually cause a sensitization of some tissues of a susceptible host to the bacteria and their products of metabolism. Such sensitization is based upon a hereditary and constitutional predisposition, and manifests itself in

one or another type of clinical syndrome depending upon the hereditary and constitutional character of the individual. This allergic mechanism (bacterial hypersensitiveness) is probably similar to that of tuberculosis.

These patients having active rheumatoid arthritis have been studied bacteriologically from this viewpoint, and only the strains yielding potent soluble toxins have been selected. These toxins were modified with formalin and treatment by desensitization was carried out, usually lasting from 3 to 6 months. The percentage of clinical improvement even in advanced cases with deformities is most gratifying.

The life cycle of the various types of arthritic patients as well as the intradermal tests with bacterial toxins and their importance both in diagnosis and as a guide in therapy will be demonstrated.

M49. The Chemistry of Diphtheria Toxin. MONROE D. EATON, Department of Bacteriology, Yale University School of Medicine, New Haven, Conn.

Diphtheria toxin was purified by precipitation with ammonium alum in the presence of one-third saturated ammonium sulphate solution, dissolving in sodium citrate and precipitating twice with cadmium chloride in the presence of sodium citrate, using sodium bicarbonate to elute the toxin from the cadmium precipitates.

The toxin was prepared on the protein-free proteose peptone medium of Wadsworth and Wheeler. With progressive purification the percentage of protein nitrogen in the toxic fraction increased. About 99.5 per cent of the nitrogenous impurities were separated from the toxin. The purified product contains 80 to 90 per cent of coagulable protein and a residuum of non-protein nitrogenous impurities. The M.L.D. of the purified toxin is 0.0001 to 0.0002 mgm. and the Lf unit contains about 0.0005 mgm. of N.

Diphtheria toxin is precipitated by all of the reagents which precipitate proteins. It has not been possible to separate the toxin from protein by repeated precipitations and adsorptions comprising a considerable variety of methods. Bacterial protein fractions colored red and green have been separated from the toxin which is colorless. The toxin is not carried down in a precipitate of bacterial protein produced in the crude filtrates by one-third saturation with ammonium sulphate. However, the purified product contains traces of bacterial protein detectable by precipitin tests with anti-bacterial serum.

The purified toxin gives definite biuret and other protein reactions only in solutions containing more than 100 Lf units of 2,000 M.L.D.

per cubic centimeter. Tests for tryptophane are very weak and those for sulphur, carbohydrate, and lipoid, negative. Phosphorous may be present either in the toxin or in an impurity.

The toxin appears to be a protein of more complex structure than the proteoses in the medium on which it was formed. This would indicate that it is not a protein split product but a protein synthesized by the diphtheria bacillus. The results of this work indicate also that the immunological and physiological properties of diphtheria toxin are all due to a single substance.

M50. The Effect of Combination with Diazo Compounds on the Immunological Reactivity of Antibodies. HARRY EAGLE, DOROTHEA EGLESTON SMITH AND PERCY VICKERS, University of Pennsylvania Medical School.

Sufficient coupling with diazo compounds eventually destroyed the reactivity of all the antisera here studied. This was due to the progressive and simultaneous decrease in the reactivity of all the antibody molecules, rather than the inactivation of an increasing proportion of molecules. The rate of inactivation varied among the several antisera. By stopping the reaction at intervals, it was possible to prepare partially inactivated antisera of peculiarly modified reactivity.

Thus, the flocculating activity of diphtheria antitoxin with toxin was completely destroyed long before there was any demonstrable impairment of its protective titer *in vivo*. The first change induced in antipneumococcus horse sera was the complete loss of reactivity with the capsular carbohydrate at a time when the agglutinating, animal protecting and complement fixing activity of the sera were only slightly affected. On further coupling, the sera no longer caused visible agglutination; but aggregation of the serum-treated bacteria could be induced by centrifugation. Still further coupling destroyed all antibody activity.

Rabbit antisera to egg albumen and horse serum no longer precipitated the homologous antigen after treatment with diazo compounds, due to their failure to combine with the antigen. The hemolytic, complement fixing and lipoid flocculating activity of coupled rabbit antisera to sheep red blood cells fell off in parallel; the hemagglutinin seemed somewhat more resistant. The reagin of syphilitic serum was destroyed almost instantaneously by comparatively small amounts of diazo compounds. Finally, in the case of antityphoid agglutinin, the isoelectric point of the coupled antibody, measured on the surface of

specifically sensitized bacteria, was found to shift from an original value of pH 4.7 to one of less than pH 2.7 as progressively more sulfanilic acid radicals added on to the antibody molecule.

The groups in protein which participate in its reaction with diazo compounds probably include aliphatic amines, the NH groups of histidine, tryptophane, proline and hydroxyproline, and the tyrosine OH. The properties of the coupled antibody suggest the extent to which these groups participate in the combination of antibody with antigen, and the extent also to which they may be responsible for the characteristic properties of the antigen-antibody compounds.

M51. The Immunological Properties of an Artificial Carbohydrate-Protein Antigen Containing Glucuronic Acid. WALTER F. GOEBEL, Rockefeller Institute.

An artificial glucuronic acid—protein antigen, prepared by combining the diazonium salt of the p-amino-benzyl β glycoside of glucuronic acid with foreign protein, has been found to react in dilutions of 1 part in 1 million with antipneumococcus horse sera, types III and VIII. A corresponding antigen containing glucose is serologically inert, however.

It has been shown that glucuronic acid is a constituent common to the specific polysaccharides of types III and VIII pneumococcus, and it has been suggested that the chemical basis for the immunological cross reactions exhibited for these microörganisms resides in the configuration of the uronic acid constituent of the specific soluble substances.

It is believed, furthermore, that the immunological activity of the artificial glucuronic acid-protein antigen in type III and VIII antipneumococcus sera can be attributed to the interaction of uronic acid antibodies, elicited by the highly polar uronic acid constituents of the bacterial polysaccharides, with the uronic acid radical of the artificial antigen.

Different species of animals are being immunized with the artificial glucuronic acid protein antigen in order to determine whether the corresponding antisera possess bactericidal properties.

M52. Hydrogen-Ion Concentration in the Preparation of Tuberculoprotein Antigen. ERNEST B. HANAN AND SOPHIA ZURETT, Buffalo City Hospital and University of Buffalo Medical School.

After studying many different methods for the isolation and concentration of the antigenic principle of tuberculoprotein, it was observed

that adjustment of hydrogen-ion concentration offered promise of being a practical and simple method. The preliminary experiments were carried out on an attenuated H37 strain of *Mycobacterium tuberculosis* grown on Long's synthetic medium.

By using standard buffer solutions it was found that the optimum pH for extraction of the tuberculoprotein from the organism without serious deterioration of antigenic property was pH 7. The optimum pH for precipitation of the protein was pH 2.8 with a range between pH 2.6 and 4.6. The optimum pH was considered as the point at which flocculation first appeared followed by maximum precipitation.

With the virulent strains of tubercle bacilli, the optimum pH for extraction was slightly on the alkaline side. The optimum pH for precipitation was pH 2.8, with a more narrow range, between pH 2.6 and 3.3.

Similar experiments with the unheated tuberculoprotein in Long's synthetic medium showed similar results to those obtained with the H37 strain of tubercle bacillus.

Addition of trichloracetic acid to the pH 2.8 filtrate produced only a slight cloudiness.

The "pH 2.8 tuberculoprotein antigen" may be further purified by resolution at pH 7 and reprecipitation at pH 2.8. It is readily soluble in distilled water made slightly alkaline with sodium bicarbonate. Following dialysis against neutral distilled water it is a satisfactory antigen for complement fixation tests for tuberculosis. The antigen is not lytic or anticomplementary, has good keeping qualities, and gives specific fixation of complement comparing favorably with Wadsworth's dialyzed distilled water extract antigen.

M53. A Preliminary Study of the Absorption Spectra of Antigens. M. O'L. Crowe, Division of Laboratories and Research, New York State Department of Health, Albany.

Quantitative absorption curves in the visible and ultra-violet regions of the spectrum, λ 6800 to 2150 Å, have been determined for four samples of three types of beef-heart antigens. Differences are noted between the curves of the three types and between the two samples of one type. An absorption curve for purified cephalin is also presented.

M54. Further Observations on Pneumococcal Hemolysin and its Inactivation. Barnett Cohen and Harry Shwachman, The Johns Hopkins School of Medicine.

1. A systematic examination was made of the ability of the oxidants from 18 well defined reversible oxidation-reduction systems to inactivate the lysin at pH 7.6. It was found that all the oxidants of systems with an E_h' of -0.07 volt or higher (more electropositive than K_4 indigo trisulfonate) on the oxido-reduction scale of potential can be made to inhibit the hemolysin *reversibly*. Those more electronegative did not, although two of the latter produced some irreversible inactivation, an effect due apparently to specific reaction or precipitation. (A study of the reductants of the above systems reveals interesting peculiarities which will be reported later.) The results indicate not only the zones of reduction potential in which the lysin may be active or inactive, but also that it may be possible to define a level of reduction potential characteristic for the pneumococcus (type II) hemolysin—a reversible oxidation system with the characteristics of an enzyme.

2. It is important to emphasize that the *rates* of reversible inactivation vary considerably for the different reagents and are not necessarily in the order of the positions of their systems on the scale of potential.

3. We have been able, by suitable treatment, to reverse the inhibitive effect of cholesterol on the lysin.

4. The evidence presented last year (Jour. Bact. **29**: 54) suggesting that the lytic activity is associated with the presence of a thiol grouping has been augmented by the following additional observations. Examination of concentrated extracts shows the presence of material which is nitroprusside-positive. Moreover, iodoacetate ion can be made to inactivate the hemolysin reversibly.

M55. Chemical and Immunological Properties of Bacterial Proteins. MICHAEL HEIDELBERGER, ARTHUR E. O. MENZEL AND FORREST E. KENDALL, Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

Methods, based on extraction by successive buffer solutions of increasing alkalinity, are described for the isolation and fractionation of proteins of the hemolytic streptococcus and of the tubercle bacillus group. Chemical and immunological differences between the fractions are discussed, as is also the question of the nomenclature of bacterial proteins, their relation to nucleoproteins, and the simultaneous formation of antiprotein and anticarbohydrate when bacterial proteins are injected into animals.

M56. The Lipoid Content of Immune Aggregates. FRANK L. HORSFALL, JR., AND KENNETH GOODNER, Hospital of the Rockefeller Institute.

Precipitates formed by the interaction of antipneumococcus horse or rabbit sera with type I pneumococcus capsular polysaccharide have been found to contain lipoids characteristic of the species orgin of the antibody. The lipoid analyses will be discussed with general reference to the relationship of lipoids to immunological reactions.

M57. Studies of the Immunizing Antigen of Pneumococcus Types I and II. LLOYD D. FELTON AND BENJAMIN PRESCOTT, The Johns Hopkins University, Baltimore, Maryland.

One sample each of Type I and Type II pneumococcus polysaccharides was studied to determine chemical and immunological changes brought about by N/10 NaOH at 100°C. and by ammonium hydroxide at 4°C. In the case of Type II, the chemical study showed decrease in the amount of acid found in an acid distillate, complete destruction of -oxyl group, an increase in the glucose number. These changes were followed by increased combining activity with serum, with no significant change in other immunological tests. With Type I significant changes were found in both chemical and immunological tests. There was a drop in the amount of acid in an acid distillate from 7.3 to 0.8 per cent, in the -oxyl per cent from 2.9 to 0, in bisulfite addition in a 10 mgm. sample from 11.9 to 0.23 cc., in iodine addition from 1.3 to 0.03 cc., and in glucose number from 15.6 per cent to 0. There was no appreciable change in nitrogen content. A decrease was observed in precipitin titer from 1:640,000 to 1:80,000 with NaOH treatment, and an increase to 1:2,500,000 with ammonia treatment. The immunizing action decreased from 1:25,000,000 to 1:1,000,000 with NaOH treatment, and increased to 1:100,000,000 with ammonia treatment. The optical rotation varied from $[\alpha]_D = +150$ in the control material to 0 in the NaOH treated, and up to +170 in the ammonia treated. Further, the heat treated sample on subsequent treatment with ammonia at icebox temperature, showed an increase in precipitin titer from 1:80,000 to 1:1,250,000, and the optical rotation from 0 to +100.

M58. Is Immunological Aggregation (Flocculation) Specific? SANFORD B. HOOKER, Evans Memorial, Boston.

It has been shown that the velocity of aggregation, in the region of

1. A systematic examination was made of the ability of the oxidants from 18 well defined reversible oxidation-reduction systems to inactivate the lysin at pH 7.6. It was found that all the oxidants of systems with an E_o' of -0.07 volt or higher (more electropositive than K_3 indigo trisulfonate) on the oxido-reduction scale of potential can be made to inhibit the hemolysin *reversibly*. Those more electronegative did not, although two of the latter produced some irreversible inactivation, an effect due apparently to specific reaction or precipitation. (A study of the reductants of the above systems reveals interesting peculiarities which will be reported later.) The results indicate not only the zones of reduction potential in which the lysin may be active or inactive, but also that it may be possible to define a level of reduction potential characteristic for the pneumococcus (type II) hemolysin—a reversible oxidation system with the characteristics of an enzyme.

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Precipitates formed by the interaction of antipneumococcus horse or rabbit sera with type I pneumococcus capsular polysaccharide have been found to contain lipoids characteristic of the species orgin of the antibody. The lipoid analyses will be discussed with general reference to the relationship of lipoids to immunological reactions.

M57. Studies of the Immunizing Antigen of Pneumococcus Types I and II. LLOYD D. FELTON AND BENJAMIN PRESCOTT, The Johns Hopkins University, Baltimore, Maryland.

One sample each of Type I and Type II pneumococcus polysaccharides was studied to determine chemical and immunological changes brought about by N/10 NaOH at 100°C. and by ammonium hydroxide at 4°C. In the case of Type II, the chemical study showed decrease in the amount of acid found in an acid distillate, complete destruction of -oxyl group, an increase in the glucose number. These changes were followed by increased combining activity with serum, with no significant change in other immunological tests. With Type I significant changes were found in both chemical and immunological tests. There was a drop in the amount of acid in an acid distillate from 7.3 to 0.8 per cent, in the -oxyl per cent from 2.9 to 0, in bisulfite addition in a 10 mgm. sample from 11.9 to 0.23 cc., in iodine addition from 1.3 to 0.03 cc., and in glucose number from 15.6 per cent to 0. There was no appreciable change in nitrogen content. A decrease was observed in precipitin titer from 1:640,000 to 1:80,000 with NaOH treatment, and an increase to 1:2,500,000 with ammonia treatment. The immunizing action decreased from 1:25,000,000 to 1:1,000,000 with NaOH treatment, and increased to 1:100,000,000 with ammonia treatment. The optical rotation varied from $[\alpha]_D = +150$ in the control material to 0 in the NaOH treated, and up to +170 in the ammonia treated. Further, the heat treated sample on subsequent treatment with ammonia at icebox temperature, showed an increase in precipitin titer from 1:80,000 to 1:1,250,000, and the optical rotation from 0 to +100.

M58. Is Immunological Aggregation (Flocculation) Specific? SANFORD B. HOOKER, Evans Memorial, Boston.

It has been shown that the velocity of aggregation, in the region of

excess antibody, is a linear function of the concentration of antigen; this conforms mathematically with von Schmoluchowski's theory of colloidal flocculation. This relationship has been used to test Marrack's "lattice" theory that the *aggregation* of particles is specific and due to the link provided by further antigen or antibody molecules.

The homologous reactants of two antigenically independent systems were so proportioned that visible particulation occurred at about the same time in corresponding tubes of the two series of tests. When tested in mixtures, of the same final concentrations, it was found that particulation was accelerated in direct proportion to the increase in the number of reacting antigenic molecules. This may be interpreted to indicate that the antibody-coated molecules of each antigen enter indifferently and nonspecifically into the formation of the mixed precipitate.

M59. Laboratory Diagnosis of Blastomycosis by Culture and by Complement Fixation. DONALD S. MARTIN AND DAVID T. SMITH, Duke University Medical School, Durham, N. C.

A review of the literature shows numerous cases in which budding organisms were present in biopsy or necropsy sections, but the diagnosis could not be confirmed by culture. Cultures are necessary because there are several types of fungi which appear as round bodies in the tissues.

We have isolated *Blastomyces dermatitidis* from 8 consecutive cases of generalized Gilchrist's disease. Cultures were obtained from all of these cases on ordinary blood agar plates, but the organisms would not grow on Sabouraud's medium unless the original inoculum contained blood or pus. We have maintained blastomycetes in the yeast-like stage for as long as 4 years by subculture on blood agar slants. When subcultured on Sabouraud's medium, aerial hyphae develop, but a reversion to the yeast-like form occurs when replanted on blood agar.

The sera of 5 out of 6 patients with generalized Gilchrist's disease contained complement-fixing antibodies for 7 strains of *Blastomyces dermatitidis*. This offers the possibility of diagnosis by complement-fixation when there is no material available for culture or biopsy.

M60. Studies on the Production of Toxin by Clostridium histolyticum. SARAH ELIZABETH STEWART, National Institute of Health, Washington, D. C.

A toxin of high potency was produced by culturing *Clostridium*

histolyticum in 2 per cent glucose broth with a pH of 7.0 to 7.3 to which 5 per cent horse serum had been added. The pH of the medium and the period of incubation were shown to be of importance. The optimum pH range for toxin production was found to be from 7.0 to 7.4. When the pH exceeded 7.4, the potency of the toxin was found to decrease; at a pH 7.6 to 7.8 no toxin production was demonstrated. Fifteen hours incubation at 37°C. was found to give a toxin of high titer. With an increase in the time of incubation a decrease in toxicity was observed; this increase in incubation time was accompanied by an increase in pH. After 24 hours incubation the pH was found to be 7.8 to 7.9.

Glucose added to the broth was found to increase the potency of the toxin, although *Clostridium histolyticum* is known to be nonsaccharolytic. This increase in toxicity was shown to be due to the production of a hemolysin. Fifteen hour cultures on the above medium without glucose did not show any hemolysis of a 5 per cent suspension of washed red blood cells even in 0.5 cc. amounts, while the glucose cultures gave complete hemolysis with 0.16 cc. It was not possible to demonstrate that the glucose was utilized as no appreciable decrease in the percentage of glucose could be shown after culturing for 15 hours. Quantitative methods for sugar determinations were used. The part played by the glucose has not been determined as yet.

Glucose in the culture medium did not increase appreciably the proteolytic enzyme. This lytic property was measured on milk agar plates. The proteolytic enzyme appears to be independent of the exotoxin. Formalized toxins which were found to be non-toxic showed no decrease in proteolysis.

M61. Heat Stable and Heat Labile Antigens in the Botulinus and Related Groups of Spore-Bearing Anaerobes.[1] L. S. McClung, Research Division, American Can Company, Maywood, Illinois.

In an investigation of the agglutination reactions of the botulinus-parabotulinus groups of spore-bearing anaerobes a study was made of about 160 cultures isolated over a period of years and from source material of wide geographic origin. The sub-groups of the Type A and B cultures established in 1925 by Schoenholz and Meyer were found to be dependent upon the reactions of the heat labile fraction. All

[1] These experiments were done in the laboratories of the Hooper Foundation for Medical Research and the Department of Medical Bacteriology of the University of California through the courtesy of Dr. K. F. Meyer.

proteolytic strains (of both Type A and B) were found to possess the same heat stable antigen, regardless of the previous group history or geographic origin of the strain, as shown by extensive cross reactions and reciprocal absorption tests. Cultures similar in morphology and physiology, but which were non-toxic and which had previously been identified by positive cross reaction in the type sera (labile antigen) of one of the established groups, were shown to possess the same heat stable antigen as the above toxic strains. Three non-proteolytic type B strains (group VII—unclassified—on heat labile fraction) were uniform in giving negative results in the serums prepared against heat-treated antigens of the proteolytic strains; similarly little or no reaction was obtained with the proteolytic type culture antigen in the serums of these strains. The antigens of the types C and D cultures also appear distinct but the number of available representatives of these types limits conclusions as regards the inter-relationship of these. The data of cross reactions and absorption tests are given for non-toxic mesophilic anaerobes including *Clostridium sporogenes*, *Clostridium parasporogenes*, *Clostridium bifermentans* and unclassified cultures.

M62. A New Method of Preparing Antigen for Serological Tests in Syphilis. AUGUSTUS WADSWORTH AND RACHEL BROWN, Division of Laboratories and Research, New York State Department of Health, Albany.

The preparation of antigens for the serodiagnosis of syphilis was investigated in order to develop a procedure by which the fresh tissue would undergo little change and still yield antigenic extracts capable of ready duplication. Fresh, ground beef-heart tissue was infused at 55°C. with a 20 per cent sodium chloride solution, the whole then treated with acetone, filtered, pressed, and dried in a current of air. The treatment with 20 per cent sodium chloride reduced bacterial decomposition and so altered the tissue that it could be extracted directly with alcohol, without preliminary ether extraction, to give an antigen suitable for precipitation tests.

The sensitivity of such an antigen could be adjusted, first, by cholesterolization to the desired degree and, second, by the proper dilution with a special buffered dextrose salt solution. The addition of 20 per cent dextrose to the diluent, buffered to pH 6.0 and containing 2 per cent electrolytes, so affected the dispersion of the cholesterolized antigen that the particles were more readily soluble in salt solution and in non-syphilitic serum; it could, therefore, be used in higher concentration than in the absence of the sugar.

In a 1-tube reaction, the same antigen extract, differently adjusted by these two means, has been employed in a preliminary test of high sensitivity, giving an approximate agreement with routine complement-fixation tests of 90.1 per cent; and in a procedure comparable in sensitivity to that of the complement-fixation test, an approximate agreement of 92.8 per cent. Prozone reactions occurred which indicate that such a test cannot be given a quantitative evaluation in all instances without further study of the specimen.

M63. Conditions Affecting the Production of Toxin and "Porphyrins" by the Diphtheria Bacillus. MARY W. WHEELER AND M. O'L. CROWE, Division of Laboratories and Research, New York State Department of Health, Albany.

Removal of the "porphyrins" from diphtheria toxin without appreciably altering the potency of the toxin has been accomplished by treatment with charcoal but not by treatment with any other adsorbent tried. It has not yet been possible either to elute the porphyrins from the charcoal or to demonstrate their presence on this substance. Filtrates of cultures grown in medium containing charcoal are highly toxic and are practically free from porphyrins.

In tests made at 24-hour intervals on cultures grown in infusion-free peptone medium, the presence of porphyrins was observed as soon as demonstrable amounts of toxin appeared.

Certain differences were noted in the absorption spectra of ether extracts from toxic filtrates of cultures grown under atmospheric conditions, under reduced oxygen tension, and in an atmosphere of carbon dioxide.

Porphyrins extracted from a diphtheria toxin and added as a filter-sterilized solution to infusion-free peptone medium, in the amount used had no apparent effect on the growth or toxigenic activities of toxigenic or nontoxigenic cultures of the diphtheria bacillus. The porphyrins could be demonstrated (after incubation for as long as ten days) in the uninoculated control medium to which they had been added.

M64. The Effect of Purified Diphtheria Toxin on Planaria maculata and Paramecium caudatum. GRACE M. SICKLES AND MYRTLE SHAW, Division of Laboratories and Research, New York State Department of Health, Albany.

Toxic filtrates of *Bacillus diphtheriae* of different potency prepared

in infusion-free peptone medium were tested by immersing *Planaria maculata* in the toxin diluted beyond the point at which the uninoculated control broth, readjusted to the hydrogen-ion concentration of the toxin, proved injurious. The toxicity of the filtrates for these forms was in direct relationship to their toxic potency as determined by tests in guinea pigs. Heating the toxic filtrate or using it in combination with antitoxin did not lessen the injurious effects on the test species.

These tests were repeated with slight modifications using *Paramecium caudatum* as the test animal. The results were in general the same. The zone of toxicity of the diphtheria filtrates beyond that of the control broth was, however, much narrower.

The residual toxins of high titer, after ultrafiltration and washing to remove other metabolic products, proved to be completely nontoxic for the planarians and paramecia, while the ultrafiltrate, nontoxic for guinea pigs, was as toxic for the planarians and paramecia as was the original material.

M65. Human Sensitization to the Purified Protein Derivative of the Avian Tubercle Bacillus. JANET MCCARTER, H. R. GETZ AND R. H. STIEHM, Departments of Agricultural Bacteriology, Medical Bacteriology, and Student Health, University of Wisconsin.

A part of the Wisconsin cattle which have reacted to mammalian tuberculin have been shown to be infected with avian tubercle bacilli. Eighteen per cent of Wisconsin hogs are retained because of tuberculosis, over 99 per cent of which is caused by the avian tubercle bacillus. One-third of the flocks of chickens in Wisconsin are infected with the avian tubercle bacillus. These facts suggested that humans in Wisconsin would have opportunities to become sensitized to the avian tubercle bacillus and further that such sensitization might account for some of the reactions to mammalian tuberculin.

All freshmen entering the University are tested with tuberculin and those giving positive reactions have chest x-rays taken. This fall 500 of the freshmen men were tested with the purified protein derivatives of both the human and the avian tubercle bacilli, supplied by the kindness of Florence Seibert of the Henry Phipps Institute. Approximately one-half were negative to both tuberculins, one-fourth reacted to both, and one-fourth reacted to avian tuberculin only. Only 5 individuals reacted positively to human and negatively to avian tuberculin. The x-rays of those reacting only to avian tuberculin revealed

no evidence of pulmonary tuberculosis in about 70 per cent, some pulmonary pathology whose tuberculous nature could be questioned in about 25 per cent, healed primary tuberculosis in about 5 per cent, and no cases of adult tuberculosis. The x-rays of those reacting to both tuberculins with equal or less reaction to the avian tuberculin, showed no evidence of pulmonary tuberculosis in slightly less than 35 per cent (half of the percentage of negatives in the group positive to avian tuberculin only), some pulmonary pathology of questionable tuberculous nature in slightly more than 30 per cent, healed primary tuberculosis in slightly less than 35 per cent, and adult tuberculosis in two cases. The number of cases (20) of those reacting to both tuberculins but in greater degree to the avian, and of those reacting to human tuberculin only (5), is too small for the data to be significant. Further details on the technic of testing, on the reactions obtained, on the history of the patients, and on the x-ray findings will be presented.

No conclusions can be drawn from the data as to the significance of the reactions to avian tuberculin in indicating either sensitization to the avian tubercle bacillus or infection with the same agent. Our phenomena make manifest the fact that further work on this problem should be done, and point the direction in which it might proceed. We cannot support the contention of Fenger, Mariette, Hutchinson, and Ouellette, that there is no type specificity of the proteins from various acid-fast organisms including the human and the avian tubercle bacilli.

M66. Antigenic Relationships of a New Cyclostage (The Critical R) of the Tubercle Bacillus. RALPH R. MELLON, PHILIP J. ALMADEN AND RUTH D. RICHARDSON, The Western Pennsylvania Hospital Institute of Pathology.

This new acid-fast variant is designated as critical because it may be dissociated in two diametrically opposite directions: first, into the acid-fast S; and second, into the nonacid-fast diphtheroidal stage. It was first derived experimentally from the latter (Proc. Soc. Exp. Biol. and Med., **30**, 1, p. 110, October, 1932), but has also been obtained from the normal, virulent S-form, leaving no doubt as to its place in the tubercle cycle. It may be distinguished from other R forms described (Kahn, Petroff, and others) by its decidedly saprophytic growth characters, by the *non-caseating* character of its epithelioid lesions, failing which the reaction in animals is frankly pyogenic, and by a partially specific tuberculin. That is to say, the antigenic complex of the S includes the R, but the R has no perceptible S antigen. These Critical

R forms have been obtained from diverse sources, particularly from avian and human strains. Regardless of origin, they fall into a fairly homogeneous group as adjudged by the sensitization test. The dissociation of such a strain from nonacid-fast strains isolated from a non-caseating form of human tuberculosis (sarcoid of the skin) throws new light on the pathogenesis of this anomalous condition. Moreover, the possible rôle of such variants in that clinical and roentgenologic entity known as epituberculosis is opened for further study.

M67. Potency of Typhoid and Paratyphoid Vaccines when Freshly Prepared and After Storage, and Agglutinin-Response to Small Intracutaneous Doses. LUCY MISHULOW, ISABELLE MOWRY AND ANNE K. STOCKER, Bureau of Laboratories, New York City Department of Health.

Vaccines were tested when freshly prepared and at intervals after storage for several years, to determine whether typhoid, paratyphoid A and paratyphoid B vaccines deteriorate when they are stored in the ice-box at 8 to 10°C.

1. Rabbits were injected with the respective vaccines and their serums were tested for agglutinins. There was no deterioration in the antigenicity of the vaccines during the period of the test. 2. Mice were given one injection of the respective vaccines and were tested 10 days later with a multiple of the killing dose of virulent typhoid and paratyphoid bacilli. The same amount of protection was obtained with the stored as with the freshly prepared vaccines.

Tests were made to determine the agglutinin response to small intracutaneous doses of mixed typhoid, paratyphoid A, and paratyphoid B vaccines. A group of laboratory workers was given a total dose of 750 million bacteria (0.5 cc.) intracutaneously in 4 injections at weekly intervals. The vaccine had been stored for periods of 3 months to 1.5 years. The small intracutaneous dose gave as high as and in some instances a higher agglutination titre than a much larger intramuscular dose. There were no systemic reactions except in one case in which there was a glandular involvement which cleared up in 2 days.

Conclusions: 1. Potent vaccines may be stored in the ice-box for a period of years without any detectable deterioration, as shown by their ability to stimulate agglutinins in rabbits and to protect against a multiple killing dose of virulent cultures. 2. Intracutaneous injection of a small dose of typhoid-paratyphoid vaccine may be substituted

for the larger intramuscular or subcutaneous dose. This is in accord with the findings of Tuft, Yagle and Rogers (J. Inf. Dis., **50**: 98–110, 1932).

M68. The Significance of Age of Rabbits in the Elicitation of the Shwartzman Phenomenon. ERNEST WITEBSKY AND ERWIN NETER, Bacteriological and Serological Laboratories, Beth Israel Hospital, New York, N. Y.

This paper deals with the questions whether the lack of reactibility of young rabbits regarding the elicitation of the Shwartzman phenomenon is a regular one and at which stage during the course of their development the Shwartzman refractory young rabbits become positive.

Highly effective *Bacillus typhosus* and meningococcus "agar-washing" filtrates were used. One-tenth to 0.2 cc. of the respective filtrates was injected intracutaneously and 24 hours later 50 to 100 units in the volume of 0.5 to 1 cc. were given intravenously.

Out of 16 litters with 78 young rabbits, 1.5 to 5 weeks old, 12 litters with 58 animals did not display any reaction. The remaining 20 young rabbits belonged to 4 litters only. Two of them (3 and 5 weeks old, respectively) with 9 animals were altogether Shwartzman positive. The two other litters (2 weeks and 4 to 5 weeks old, respectively) consisted of 11 animals: 3 of them were Shwartzman negative, one showed a doubtful reaction and 7 were positive. Thus a remarkable percentage of young rabbits is refractory towards the elicitation of the Shwartzman phenomenon.

The Shwartzman negative litters were again tested after periods of one to two weeks with the same or with heterologous toxins. The same amount of toxin was used as in the first experiment, irrespective of the increased weight of the young rabbits. Many, but not all, of the originally negative animals were Shwartzman positive at the second, sometimes only in the third or fourth repetition of the test. Thus there exists a correlation between the development of rabbits and their ability to react towards the elicitation of the Shwartzman phenomenon.

M69. On the Presence of Fibrinolytic Substance in the Spinal Fluid of Patients with Streptococcus Meningitis. EDWIN NETER AND E. WITEBSKY, Bacteriological and Serological Laboratories of the Beth Israel Hospital, New York, N. Y.

Hemolytic streptococci produce a fibrinolytic agent when cultured

in glucose broth. The experiments to be reported here are concerned with the question whether the fibrinolytic substance of streptococci can also be found *in vivo*.

The spinal fluid was examined in three cases of purulent meningitis in which the culture revealed the presence of hemolytic streptococci. All three specimens displayed fibrinolytic potency. On the other hand, normal spinal fluid (25 specimens) as well as the spinal fluid from patients with meningitis due to organisms other than streptococci (1 case of pneumococcus Type I meningitis, 2 cases of meningococcus-meningitis and 1 case of tuberculous meningitis) did not show any fibrinolytic activity. On the contrary, normal spinal fluid inhibited the fibrinolytic action of effective streptococcus culture filtrates. This corresponds to the inhibitory effect of normal serum towards fibrinolysis.

The spinal fluid of one of the cases with streptococcus meningitis was filtered through a Berkefeld filter immediately after the spinal tap was performed. The filtrate also proved to be effective.

The production of the fibrinolytic substance is not limited to hemolytic streptococci alone. Therefore further studies are necessary to determine whether the fibrinolytic agent may be encountered in cases of meningitis due to other microörganisms which are able to produce the fibrinolysin *in vitro*.

M70. Variation in Streptococcus hemolyticus—Groups A to G. M. H. Dawson, Gladys Hobby and Miriam Olmstead, College of Physicians and Surgeons, Columbia University and the Presbyterian Hospital, New York City.

Lancefield's demonstration of distinct serological groups within the hemolytic streptococci has greatly simplified the study of variation phenomena within this bacterial species. Last year two of the authors presented before this Society a study on variation in Group A organisms. Since then the investigation has been extended to include Groups B to G. The results reveal the existence of a parallel dissociation pattern in all groups. Three chief variant forms—Mucoid (M), Smooth (S) and Rough (R)—have been identified in each group. The salient characteristics of each of these variant forms will be described and it will be shown that the variation phenomena exhibited by the several groups of hemolytic streptococci parallel those of a wide variety of other bacterial species.

M71. Studies of Streptococcus zymogenes. WILLIAM J. ELSER AND RUTH ALIDA THOMAS, Department of Applied Pathology and Bacteriology, New York Hospital and Cornell University Medical College.

Streptococcus zymogenes, which was first described under the misnomer *Micrococcus zymogenes* by MacCullum and Hastings in 1896, has attracted relatively little attention and in more recent years has been eliminated entirely from consideration in the leading textbooks of bacteriology. Our interest in this organism was aroused by the fact that it is not infrequently encountered in the blood of patients suffering from a subacute form of endocarditis, indistinguishable either clinically or pathologically from that produced by *Streptococcus viridans*. Our assumption that these two organisms are more or less closely related was further supported by the fact that they produce similar changes in red blood cells. We have in our possession 17 strains which have been collected over a period of years and identified as *Streptococcus zymogenes* principally on the basis of their action on gelatin and litmus milk and their colony growth on a special casein agar plate. Eight strains were recovered from the blood, 6 in cases of subacute endocarditis and 2 in arthritis, 3 from the urine in pyelitis, 4 from appendices, one from a normal throat and one from the cervix of a guinea pig. Although these strains may be divided into two groups differing in the rate at which they ferment the lactose in litmus milk cultures, when grown in the blood agar plate all produce a greenish discoloration of the red blood cells. These latter findings differ from those of both Frolisher and Denny (Jour. Bact., **16,** p. 301, 1928), and Torrey and Montu (Jour. Inf. Dis., **55,** p. 340, 1934) who claim that the majority of the strains studied by them are hemolytic, and from those of Sherman and Stark (Jour. Bact., **22,** p. 275, 1931) who differentiate *Streptococcus zymogenes* from *Streptococcus liquefaciens* because of its hemolytic activity.

Recently studies were made comparing our strains and two of the hemolytic type isolated by Dr. Torrey from stool cultures. Two hemolytic strains of *Streptococcus fecalis* obtained from the American Type Museum under this designation were also included since culturally they were apparently *Streptococcus zymogenes*. The hemolytic strains proved to be indistinguishable culturally or biochemically from our strains except in their action on blood. Colonies on blood agar plates show partial hemolysis and no greenish discoloration of the surrounding

cells. A soluble hemolysin can be demonstrated in young serum broth cultures of these strains when rabbit or human cell suspensions are used as an indicator. All types are active producers of peroxidase which in the case of our green-producing strains raises the question as to the extent to which H_2O_2 is involved in the alteration of the color of the hemoglobin. Since agglutinin-absorption tests show the two groups to be closely related, such methods fail to differentiate the two groups of streptococci under consideration. We wish, however, to call attention to the fact that in our experience all the strains recovered in pure culture from the blood in cases of subacute endocarditis, where the question of pathogenicity can hardly be questioned, were of the Alpha type. In addition, it should be emphasized that *Streptococcus zymogenes* is frequently not recognized in blood cultures since while the crowded colonies on the initial blood plates closely resemble the viridans, those on the more sparsely seeded plates may be mistaken for *Staphylococcus albus*. Serological tests to establish the relationship of *Streptococcus zymogenes* to other streptococci have been thus far inconclusive.

M72. The Types of Hemolytic Streptococci Concerned With Scarlet Fever and Scarlatinoid Angina. JOHN HAYS BAILEY, Municipal Contagious Disease Hospital, Chicago, Ill.

Two hundred forty-three cases of scarlet fever were cultured for hemolytic streptococci on admission to the hospital, and the hemolytic streptococci so obtained typed by Griffith's method. Of the cases typed 70.4 per cent showed infection by one of three of Griffith's types of streptococci, either types 2, 3, or 6, while 8.6 per cent were infected with two types at the time of admission. Typing of the cultured organism was not successful in 2.5 per cent of the cases. The type 6, very commonly associated in England with tonsillitis but seldom found in scarlet fever, was as common in Chicago's scarlet fever as the usual scarlet fever streptococci.

If complications be used as an index to severity of infection there is no difference in the 3 types of streptococci chiefly involved, but of the 5 cases that died in which typing of the streptococci had been done 4 were infected with type 2 streptococci. The other death occurred in a butcher dying of septicemia and surgical scarlet fever following abrasions of the hands.

One hundred sixty-one cases were cultured for hemolytic streptococci one day before release from the hospital. The majority of the types

found (77.6 per cent) were the same as in the admission cultures. Cases showing a double infection were 9.9 per cent and untyped cases 3.1 per cent of the total. Of these 161 discharge patients admission cultures had been typed in 100 instances, 81, being type 2, 3, or 6. Of 81, 48 had the same type of streptococcus on dismissal as on admission. Patients infected with a type 3 streptococcus are more likely to leave the hospital with another type of streptococcus than those infected with either type 2 or 6.

In addition to the scarlet fever cases, 11 persons (resident physicians, internes, student nurses, and hospital maids) suffering from severe sore throats were cultured and the hemolytic streptococci obtained typed. The term scarlatinoid angina is suggested for these cases. Four types of hemolytic streptococci were obtained from these 11 patients, types 2, 3, 4, and 6, type 3 being the most common. Since these individuals were all Dick negative none developed scarlet fever, although 6 cases of scarlet fever did develop from contact with one or more of the internes.

M73. A Comparative Study of Human and Avian Strains of Staphylococcus albus. CHARLES S. GIBBS, Massachusetts Agricultural Experiment Station, Amherst.

Staphylococcus albus appears to be commonly present in the domestic fowl either as a harmless saprophyte in the healthy fowl or as a secondary invader in outbreaks of disease such as laryngotracheitis and colds. Also it has been found to be the sole cause of bumble foot, abscesses, infection of the infraorbital sinuses, swollen wattles, pyemia, and septicemia.

Staphylococci isolated from lesions in man present the same cultural characteristics as those isolated from the domestic fowl. Cross agglutination and absorption tests are difficult to interpret because of prozones and other irregularities. However, human strains of staphylococci may be differentiated from avian strains by means of precipitin and wattle tests.

M74. Serologic Grouping of Staphylococci. JOHN E. BLAIR AND FRANCES A. HALLMAN, Hospital for Joint Diseases, New York City.

A preliminary report is made of attempts to group staphylococci, on the basis of: (*a*) precipitin tests with toxic filtrates; (*b*) precipitin tests with specific soluble carbohydrates; (*c*) reciprocal agglutinin absorption tests; (*d*) animal pathogenicity. Rabbits were immunized by intradermal injection of filtrates of 10-day aerobic broth cultures

(Burky) or by intravenous injection of heat-killed bacterial suspensions (Julianelle). At present, 4 groups of staphylococci are recognized, on the basis of reciprocal agglutinin absorption tests. Three of these groups are pathogenic and one is non-pathogenic. No satisfactory results have yet been obtained with either of the precipitin tests, both of which are being further studied. The coagulase test (Gross, Chapman) may apparently serve for preliminary separation of staphylococci into pathogenic and non-pathogenic strains.

M75. Metabolic Capacity as a Factor in the Defense against Pneumococcus Infection. ARTHUR LOCKE, The Western Pennsylvania Hospital Institute of Pathology, Pittsburgh.

The ability of the non-immune rabbit to resist invasion by type I pneumococcus is proportional to its ability to warm up after chilling. Ninety-two per cent of snuffle-free, uninjured rabbits with a "warming time" of less than 32, resist penetration from an intradermal focus to the extent of having a negative blood culture at 26 hours. Equivalent resistance is exerted by 39 per cent of rabbits with a warming time between 32 and 40. Invasion is overwhelming at warming times of more than 40. Fifty-nine per cent of snuffle-free, uninjured rabbits with warming times of consistently less than 40 survive, without apparent ill effect, intravenous injections of type I pneumococci which produce the death of slower warming rabbits within 2 to 3 days. The organisms are swept from the blood, in the faster warming rabbits, during the lag before the inception of logarithmic growth.

These and other observations on the relation of warming time to capacity for resistance are presented and analyzed for their significance in the problem of the nature of the defense against infection. Preliminary mention is made of experimentation in the direction of an index of capacity for resistance to pneumococcus infection in man.

M76. Other Oxydase Positive Bacteria Found in Cultures Made for Neisseria gonorrhoeae. LUTHER THOMPSON, Mayo Clinic, Rochester, Minnesota.

During the past 8 months, 1100 cultures for *Neisseria gonorrhoeae* were made according to the method proposed by McLeod, *et al.*, in 1934 (Jour. Path. Bact., **39**: 221). Colonies of bacteria other than *Neisseria gonorrhoeae* which gave a positive oxydase reaction were encountered in 11 instances, as follows: *Neisseria catarrhalis* in two cases; *Neisseria flava* in one case; *Hemophilus influenzae* in one case; and a Gram-negative diplo-bacillus, as yet unidentified, in 6 cases. The

colonies of the last mentioned organisms resemble closely those of *Neisseria gonorrhoeae* and give a strong oxydase reaction. At present it is thought most likely that they belong to the genus *Achromobacter*.

This series does not include the members of the colon-aerogenes group, some of which give a faint oxydase reaction which soon fades. These latter are not likely to be confused with *Neisseria gonorrhoeae* on account of the much larger colonies.

The results emphasize the necessity of a microscopic examination of suspicious colonies in all cases and of isolation and study in pure culture in some instances in order to avoid an erroneous report.

M77. An Epizootic Septicemia of Young Guinea Pigs Caused by Pseudomonas caviae, n. sp. M. SCHERAGO, University of Kentucky, Lexington.

Strains of *Pseudomonas caviae* n. sp. isolated from the internal organs of young guinea pigs that died of a septicemic disease reproduced the disease when injected into young guinea pigs (200 grams or less).

The organism resembles *Pseudomonas aeruginosa* in morphology and in staining reactions except that it is encapsulated. It differs from *Pseudomonas aeruginosa* in its cultural and biochemical characteristics, producing an iridescent greyish white growth on agar slants and imparting to this medium a light greenish yellow tinge. The gelatin liquefaction is infundibuliform. The growth on potato is scanty and light yellow to light orange in color.

Acid but no gas is formed from dextrose, levulose, galactose, maltose, cellobiose, mannitol, lactose (small amount after 5–7 days), arabinose, saccharose, trehalose, sorbitol, mannose, dextrin, salicin, glycerol, and starch. Xylose, dulcitol, rhamnose, inulin, adonitol, raffinose and inositol are not attacked. Milk is acidified, coagulated and peptonized. Litmus is partially reduced. Indol and ammonia are formed and nitrates are reduced to nitrites. Hydrogen sulfide is not formed. The methyl red reaction is positive and the Voges-Proskauer negative. There is no growth in citrate broth. Methylene blue is reduced. Catalase is not produced. Blood is not hemolyzed. Blood serum is not liquefied.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. The Incidence and Significance of the Presence of Borrelia vincenti and other Spirochaetaceae on Beverage Glasses. DON CHALMERS LYONS, Jackson, Mich.

This study of public health significance was carried out with the coöper-

ation of Drs. W. L. Mallman and W. L. Chandler of the Michigan State College. A previous study of beverage glasses had revealed the presence of high numbers of bacteria on the rims of glasses cleansed by the usual methods in vogue in taverns, eating establishments, etc., where alcoholic beverages were dispensed. Technic was developed to obtain smears from the rims of these glasses which would preserve the microorganisms until a darkfield and stained smear study could be made. Such smears were made from the rinsed glasses after their return from the customers, and from glasses racked upon the bar and ready for use. Specimens were also obtained of the rinse waters.

The increasing prevalence of Vincent's stomatitis or trench mouth suggested the possibility that proper methods were not being taken in beverage dispensing establishments to prevent the spread of this disease.

This study revealed surprisingly large percentages of *Borrelia vincenti* and other **Spirochaetaceae** on beverage glasses and suggests the necessity of better control of methods of glassware cleansing in such establishments.

A2. Investigation of a Milk-Borne Epidemic of Staphylococcus Food Poisoning. HOWARD J. SHAUGHNESSY AND THOMAS C. GRUBB, Division of Laboratories, Illinois State Department of Public Health, Springfield.

An outbreak of food poisoning occurred on a small farm in the southern part of Illinois during July, 1935, which involved some 25 persons who had consumed milk, or ice cream made from the milk produced by three cows on the farm. An investigation of the milk supply revealed the following facts: While the milk from the three cows was normal in appearance and flavor, a positive brom thymol blue test was obtained on one-quarter of each cow. Aseptically collected pooled samples of milk and samples from individual quarters contained from 890 to 19,500 bacteria per cubic centimeter, the majority of which were white or yellow staphylococci, though a few green-producing streptococci were also found. Several of the staphylococcus and streptococcus strains were grown in a semi-solid medium under a partial carbon dioxide pressure and sterile filtrates of these cultures were fed and injected into *Macacus rhesus* monkeys. The filtrate of one of these strains, a hemolytic staphylococcus of the albus variety, produced vomiting in monkeys approximately two hours after feeding or injection. One cubic centimeter of this filtrate produced vertigo, nausea, vomiting, cramps and diarrhea in a human volunteer after a 4 hour incubation period.

Since no members of the *Salmonella* or dysentery groups could be isolated from the stools of several of the victims of the epidemic, and no agglutinins for these organisms were detected in their sera, it was concluded that the outbreak was due to the consumption of milk or ice cream made from the milk containing an enterotoxin elaborated by the same or similar strains of staphylococci isolated from the milk.

A3. Acid and pH Variations in Ananas comosus Merr. in Relation to Swells Caused by Clostridium Sp. C. H. SPIEGELBERG, Pineapple Experiment Station, University of Hawaii.

Bursting swells caused by 2 types of saccharolytic *Clostridium* are considered in relation to pH and acid variations of the raw fruit and of the canned product during and following the process.

The spores of the causal organisms survive processes which are generally adequate to insure commercial sterility in acid products, but are unable to germinate in pineapple juices of approximately pH 4.5 and below. This indicates that occasional cans have a pH far higher than the approximate mean of Fancy Sliced goods which is pH 3.8.

Wide variability of pH has been found in the Fancy Sliced grade. The range encountered during routine inspections was pH 3.6 to 4.4, but in packs where swells had occurred cans with pH up to 4.7 were found. Both raw fruit and syrup are considered responsible.

Methods involving visual characters of the fruit and a color indicator test paper (p-sulfo-o-methoxybenzene-azo-dimethyl-a-naphthylamine) demonstrated the occurrence of fruits with pH up to 5.2. Wide variability of pH in fruits of similar titratable acidities was noted.

Commercial syrups of pH 4.3 and 5.2 prepared from highly buffered juice, were applied to the selected fruits. Use of the low pH syrup resulted in lower pH in cans after diffusion, reducing chances of high pH cans susceptible to swells caused by *Clostridium* spp.

Experimental packs with varying pH (attained with both selected fruit and buffered syrups) show *Clostridium* swells only in cans with pH above 4.4. Prolonged processing is not feasible because of deterioration of the product. The conclusion is reached that commercial swells of this type result from a too high pH in cans during and after processing.

A4. The Preservation of Concord Grape Juice. CARL S. PEDERSON, New York State Agricultural Experiment Station, Geneva.

Concord grape juice is subject to rapid deterioration if improperly

handled. Commerical packers have attributed this loss of quality to pasteurization. In the past a high temperature of pasteurization, that is, 190°F. (87.8°C.) has been considered necessary.

The rate of killing of yeast, bacteria and mold naturally present in grape juice has been determined. Sources and types of contamination in commercial equipment have been studied. Results have shown that organisms present in Concord grape juice are readily killed at 155°F. (68.3°C.) and that pasteurization at 165°F. (73.9°C.) allows a sufficient margin for killing all organisms. An appreciation of sources or recontamination such as mold on corks or in the foam is necessary to successful pasteurization at low temperatures. Low temperature pasteurization not only results in less flavor change due to heat but also less rapid deterioration due to oxidation. Oxidation may be lessened by elimination of air.

A5. Microbiological Examination of Dried Foods. J. A. Clague and James E. Fuller, Massachusettes Agricultural Experiment Station.

The number of microörganisms on dried foods varies from a few thousand per gram as a maximum on dried fruits up to several millions per gram on dried vegetables.

Tests conducted with a small tunnel drier showed that artificial drying effectively eliminated yeasts and materially reduced the numbers of bacteria and molds on dried fruits. Reduction of the numbers of microörganisms on vegetables during the drying process was not so marked as with fruits; in fact, dehydration did not completely eliminate *Escherichia coli* which was inoculated onto the vegetables.

Solid media suggested for a study of dried foods are: plain nutrient agar as recommended in Standard Methods of Water Analysis and tomato agar for yeasts and molds.

The methods used in the commercial dehydration of foods should produce a safe product, especially when it is considered that most of these foods are cooked before being consumed.

A6. Types of Oospora Found in Butter. C. M. Sorensen and E. H. Parfitt, Purdue University.

In the examination of acidulated potato dextrose agar plates of 600 samples of pasteurized commercial sour cream butter it was noted that the *Oospora* colonies that grew on these plates differed in appearance. Cultures were made of those colonies differing in appearance and these

cultures were studied as to their morphological and cultural characteristics. Apparently 8 different varieties were obtained all of which could be characterized as *Oospora lactis*. These varieties were studied and significant differences were found within the cultures as to caseolysis, lipolysis of milk and tributyrin, growth rates, optimum temperature, and majority thermal death point. Differences were also found in their production of caseolytic and lipolytic enzymes.

A7. The Correlation between the Spoilage of Butter and the Presence of Fat Splitting and Casein Digesting Bacteria. C. N. STARK AND B. J. SCHEIB, Cornell University, Ithaca, New York.

Five series of butter consisting of 576 samples, made and held under known and controlled conditions, have been examined. At various times during the holding period the quality of the butter has been scored and the total numbers of bacteria, as well as the numbers of fat splitting and casein digesting bacteria, have been determined by plating on Nile blue sulphate agar and milk agar.

Of 486 cultures studied, those present in large enough numbers to be considered of importance in the spoilage of butter were Gram negative rods, Gram negative micrococci, Gram positive sarcina and Gram positive non-spore producing rods. Of those groups which occurred in large numbers, only the Gram negative rods were regularly present in all of the butters tested. Holding temperatures of 5°, 10°, and 24°C. were used. Unsalted butter made from sweet cream pasteurized at 73.9°C. (165°F.) for 30 minutes was found to be free from most spoilage factors. The importance of recontamination by bacteria able to grow in butter was demonstrated.

In the absence of other spoilage factors, a direct correlation seemed to exist between the numbers of fat splitting and casein digesting bacteria and the keeping quality of the butter.

Data are available to show the importance of the use of milk agar as an indication of the keeping quality of butter.

A8. The Use of Tribasic Sodium Phosphate in Cleaning Dairy Equipment. L. A. ROGERS AND FRED C. EVANS, Bureau of Dairy Industry, Washington, D. C.

A 5 per cent solution of tribasic sodium phosphate is not only an excellent detergent, but through its high pH has a distinct bactericidal action. Milking machine parts held between milkings in a 5 per cent solution are cleaner bacteriologically than when held in the usual chlorine solution.

Hand separators receiving no treatment except rinsing with water and holding submerged in a solution of tribasic sodium phosphate are physically clean and do not increase the bacterial count of the milk appreciably.

The corrosive action of the alkali can be nearly or quite eliminated by the addition to the phosphate crystals of 3 per cent of sodium chromate.

A9. The Sequence of Bacterial and Chemical Changes Occurring in Mastitis Milk. L. A. BURKEY, G. P. SANDERS AND J. F. CONE, Bureau of Dairy Industry, Washington, D. C.

Bacterial and chemical analyses of mastitis milk made at random are of limited value unless a sequence of the changes is well established. This study was made on fore-milk from individual quarters of first-calf heifers showing no evidence of mastitis at the start of the lactation. Analyses were made regularly at weekly intervals from the beginning of the first lactation and continued as long as practicable after the onset of the infection.

Results to date show: that the leucocyte count may reach millions and streptococci may be present for a considerable time before physical or chemical changes occur in the milk; that when *Streptococcus mastitidis* causes the infection it is present before and during the early stages of the disease; that the numbers of streptococci may vary or streptococci may even be absent in the later stages of the disease; that the pH of the milk does not increase until several weeks after the onset of the infection or even may decrease in the early stages, resulting in so-called acid mastitis; that a softening of the curd, a loss of rennet coagulability, and an increase in the percentage of chlorine are usually associated with a rise in the pH of the milk, which eventually follows the inception of the disease; that the casein content may or may not be decreased as a result of mastitis; and that milk yield decreases as the result of the infection and does not return to normal after all evidences of mastitis in the milk have disappeared.

A10. Observations on Staphylococci Associated with Bovine Mastitis. W. N. PLASTRIDGE, F. J. WEIRETHER AND L. F. WILLIAMS, Storrs Agricultural Experiment Station, Storrs, Connecticut.

As part of our segregation program for the control of infectious bovine mastitis, quarter samples were collected periodically from allegedly streptococcus-free groups of animals in 4 experimental herds. The

samples were examined for the presence of streptococci and staphylococci, and for laboratory evidence of mastitis. Cultures of staphylococci were obtained from streptococcus-free samples, and their characteristics determined and compared with other evidence of mastitis. Evidence of mastitis when found in samples yielding staphylococci was usually limited to a leucocyte count of 300,000 or more cells per cubic centimeter.

Of 465 cultures studied, 145 produced weakly hemolytic colonies on blood agar and hemolyzed blood broth (Group 1), 67 produced weakly hemolytic colonies but failed to hemolyze blood broth (Group 2), and 253 failed to show evidence of hemolysis in either medium (Group 3). The average leucocyte count associated with each group was as follows: Group 1—1,181,000; Group 2—925,500; and Group 3—250,000. The relation of hemolytic ability to invasive power is further shown by the duration of infection; fifty per cent of the quarters yielding Group 1 cultures, 14.3 per cent of those yielding Group 2 cultures, and 1.0 per cent of those yielding Group 3 cultures remained infected for a period of at least one year. Preliminary work with the coagulase test, using citrated cow's blood, indicated that about one-third of the Group 1 cultures were coagulase-positive, and that these cultures possessed a higher degree of virulence than cultures lacking this property.

No close correlation between the fermentative properties of the cultures studied and evidence of mastitis was found.

A11. A Study of Some Acid-Fast Actinomycetes Isolated from Soil. RUTH GORDON AND W. A. HAGAN, Department of Pathology and Bacteriology, New York State Veterinary College, Cornell University, Ithaca.

Three strains of acid-fast actinomycetes isolated from scabby potatoes and 8 from soil have been compared with two pathogenic strains isolated from human and animal lesions. No significant differences were found between the strains of the group except in pigment formation and pathogenicity.

All strains produced acid from glycerol, reduced nitrates, rendered litmus milk alkaline, and grew at 37°C. (one exception) as well as at room temperature. None grew at 47°, fermented sugars, hydrolyzed starch or cellulose, produced indol, liquefied gelatin or digested egg albumin. All produced thick pellicles on fluid media and heavy, wrinkled growths on agar, which in some instances was tough, leather-like, and adherent to the medium, and in others was friable and not

adherent. When freshly isolated all strains produced aerial hyphae, but some of the strains appeared to lose this property rather quickly. All were definitely acid-fast, although non-acid-fast elements were always present. All fragmented easily, yielding coccoid and bacillary bodies that resembled acid-fast bacteria. The strains from potatoes and soil, with one exception, produced pigment varying with the different strains from yellowish to orange. The exception was unpigmented. One of the tissue strains (*Actinomyces asteroides*) produced a yellowish pigment, the other (*Actinomyces gypsoides*) was unpigmented.

The two strains of tissue origin produced fatal infections when injected into guinea pigs. The soil and potato strains were practically non-pathogenic for this species. One of the potato strains was highly pathogenic for rabbits soon after it was isolated, but lost most of its pathogenicity within a few months of artificial cultivation. One of the tissue strains was pathogenic for rabbits, and the other was not, although it was said to have been pathogenic some years ago at the time it was isolated.

It is concluded that acid-fast actinomycetes, very closely related to well known pathogenic species, exist in soil.

A12. Industrial Utilization of Gums Produced by Fungi. J. R. SANBORN, Arthur D. Little, Inc., Cambridge, Mass.

In the increasing industrial use of vegetable gums a definite place appears for some of the gummy products formed by microörganisms. The tough pellicles produced by several species have already been used in the manufacture of commercially valuable products, including leathery, rubbery, as well as transparent sheet materials. Many types of growth possess adhesive and cohesive properties. These may be manipulated and applied to provide mucilaginous surfaces for finely divided coating materials or intermediate layers for binding sheets of widely varying types. Except for those growths which become characteristically hard and matted, the majority of the microbial gums maintain a gelatinous state. Their tenacious retention of considerable amounts of water is of primary significance in commercial applications.

Utilization of the synthetic functions of microörganisms in food preparation further enhances the importance of gum production. Formation by fungi of gelatinous forms of cellulose, sugars, albumen, fats, esters, and growth accessory factors, appears to be intimately associated with growth agglomerates. The constructive activities of yeasts and related fungi have been extensively investigated. General uses

for *Mucor*, *Rhizopus*, *Penicillium*, *Trichoderma*, *Oidium*, cellulose-forming bacteria, and certain **Polyporaceae** are also suggested. A variety of low cost basic raw materials such as waste sulphite liquor, blackstrap molasses, distillery slop, wood residues, vegetable mashes, and corn syrup, are available.

Gummy varieties of *Oidium*, now under investigation at this laboratory, produce unusually heavy yields of a hydrocellulose gum from glucose, glycerol, and mannitol. Cultivation of these organisms on a commercial basis may be accomplished in shallow tanks or ponds containing potato mash and glucose. Raw materials impose a cost of about one cent per pound on freshly prepared agglomerates containing 90 per cent water. While more or less complete removal of water would effect a substantial increase in cost, some of the uses suggested for the material undoubtedly justify this added expense.

A13. Formate Utilizing Organisms from a Trickling Filter. R. H. WEAVER, University of Kentucky.

A modification of the method proposed by Winogradsky for the study of the organisms especially adapted to the utilization of organic substances in the soil has been used to determine the organisms responsible for the destruction of formates in a trickling filter. The predominant organism found on the aerobic silica gel plates containing sodium formate as the only source of energy was a member of the *Pseudomonas* genus. An organism, apparently identical with *Bacillus methylicus* of Loew, was present in smaller numbers. In addition occasional colonies of flavobacteria and of other organisms were found. On the plates that were incubated anaerobically only colonies of members of the coli-aerogenes group were found.

A14. Influence of Host Plant on Effectiveness of Rhizobia. S. V. BOND, P. W. WILSON AND F. C. WAGNER, University of Wisconsin, Madison.

Previous studies at Wisconsin and other experiment stations have established that different strains of the root nodule organisms vary in effectiveness, i.e., in ability to fix atmospheric nitrogen in association with the proper host plant. The underlying causes of this variation are not known, but one factor appears to be the host plant specificity, i.e., a given strain may be judged effective on alfalfa (*Medicago*) but ineffective on sweet clover (*Melilotus*). The effect of different species of the same genus of host plant on the symbiosis has not been ade-

quately determined. This report deals with the differential response of various species of *Melilotus* to inoculation with a number of strains of *Rhizobium melilotii*.

In one experiment 10 strains of the organisms were used to inoculate 5 species of *Melilotus;* in two others, 5 strains of the bacteria were tested on these same 5 species of the host plant. The *Melilotus* species were pure lines furnished by the Genetics Department; the organisms were isolated from alfalfa and sweet clover plants and ranged from "ineffective" to "superior" in tests on *alfalfa*. A technique was developed for growing the plants in one-half gallon pots under a light-house covered with Pliofilm. All plants in an individual house were inoculated with the same strain of bacteria; hence, cross-contamination among the pots was eliminated.

The data shows that there is a "species variation" in host plant as well as a "strain variation" in the organisms. Not only is there a definite variation in the response of different species of sweet clover to inoculation with different strains of the organisms, but also the strain of bacteria judged most efficient may vary with the species of host plant used for the test.

A15. Comparative Efficiency of Free and Combined Nitrogen for Nutrition of the Soybean. WAYNE W. UMBREIT, F. S. ORCUTT AND P. W. WILSON, University of Wisconsin, Madison.

In biochemical studies concerned with comparison of nitrogenous compounds in inoculated soybean plants fixing atmospheric nitrogen with those in plants furnished combined nitrogen, opportunity was had to observe the relative ability of the elemental and combined forms of nitrogen to further the development of the plant. In 9 experiments made during the past 3 summers the plants were grown outside in cold-frame under conditions which afforded excellent growth. The source of combined nitrogen was usually NH_4NO_3 but in some cases $Ca(NO_3)_2$ was used.

The results of these studies may be summarized as follows:

Case 1. Excessive carbohydrate synthesis. This occurs under intense sunlight, adequate supply of CO_2, and may be favored by drought. Under these circumstances inoculated plants fail to emerge from the nitrogen hunger stage, unless combined nitrogen is added, or artificial means for reducing carbohydrate synthesis (as shading) are employed. Plants supplied with combined nitrogen develop normally.

Case 2. Optimum carbohydrate synthesis. This takes place when sun-

light is adequate but not too intense and when sufficient moisture and CO_2 are present. Under these optimum conditions the inoculated plants develop more rapidly than those supplied with NH_4NO_3. Especially during the maturity stage, the assimilation of free nitrogen is much more pronounced than is that of the fixed form.

Case 3. Sub-optimum carbohydrate synthesis. If carbohydrate synthesis is not adequate for needs of the plants, advantages of the inoculated plants over those receiving combined forms largely disappear, and little difference in the development of the plant can be observed.

Case 4. Deficient carbohydrate synthesis. If production of carbohydrate falls to a point at which there is a distinct lack of this supply of energy, once again the development of plants receiving combined nitrogen exceeds that of the inoculated plants. Apparently with a deficient supply of carbohydrate, fixation of free nitrogen can take place with less ease than can assimilation of combined nitrogen. However in this case excessive combined nitrogen is also detrimental to the development of the plant.

A16. Studies on the Root Nodule Bacteria of Certain Wild Leguminous Plants of Wisconsin. O. A. Bushnell, W. B. Sarles and E. B. Fred, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

The isolation and study of the rhizobia of the native *leguminosae* of Wisconsin has been the object of a series of varied laboratory and greenhouse investigations in which special emphasis has been placed upon the determination of the cross-inoculation group to which each of several unclassified leguminous species belongs.

This preliminary report presents the results obtained so far from the cultural and greenhouse tests applied to the 89 cultures of rhizobia isolated from 48 species of wild leguminous plants. Of these 48 species, representing 17 genera, the root-nodule bacteria of 11 have already been studied by earlier workers, leaving those of 37 "new" species to be investigated and classified. Eleven of these new species belong, apparently, to the Pea-Vetch Group, and 13 to the Cowpea Group, while 9 species requiring further study are as yet unassociated with any particular bacterial-plant groups. The other 4 new species appear to be representatives of as many minor and less common cross-inoculation groups.

A17. Experiments on the Diffusion of Nitrogenous Compounds from Healthy Legume Nodules or Roots. C. A. Ludwig and Frank-

LIN E. ALLISON, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

A. I. Virtanen and associates of Helsingfors, Finland, have recently demonstrated by rigidly controlled experiments that nitrogen fixed by legumes will diffuse outward from healthy nodules or roots and become available to non-legumes growing in close association. The present authors have conducted similar experiments, using cowpea (*Vigna sinensis*), alfalfa (*Medicago sativa*), vetch (*Vicia villosa*), and sweet pea (*Lathyrus odoratus*) growing alone and in association with various non-legumes. They were grown in jars in silica sand, using a number of inorganic nitrogen-free culture solutions, with both artificial and natural light sources, and with 0.5 per cent carbon dioxide added to the air in a few experiments.

Instead of proving beneficial, the legumes have usually depressed the meager growth of the non-legumes. In one case, a vetch-wheat culture, a slight benefit was noted but later experiments under similar conditions gave negative results. Likewise, in contrast to Virtanen's results, no soluble nitrogenous compounds could be extracted from the sand. The failure was apparently not due to inadequate aeration of the sand or to unsatisfactory light intensity. The rate of fixation in many experiments was comparable to that obtained by Virtanen; hence, the diffusion of nitrogenous compounds from legume nodules and roots does not always accompany good fixation. A number of other factors which affect growth and nitrogen fixation are being given consideration.

A18. The Stimulation of Rhizobia by Natural Humic Acid. FRANKLIN E. ALLISON AND SAM R. HOOVER, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Experiments with *Rhizobium trifolii* and *Rhizobium meliloti* are reported which show that the addition of natural soil humic acid to a synthetic (sugar-inorganic salts-nitrate) medium causes a heavy growth of these organisms. The response was measured both by direct counts, using a haemacytometer, and by oxygen consumption, according to the Warburg respiration technique. The growth obtained over the concentration range of 0–600 p.p.m. dry matter was nearly proportional to the quantity added. In the absence of humic acid no appreciable growth occurred.

The response of clover and alfalfa nodule bacteria to natural humic acid was found to be due almost wholly to its content of the essential

bacterial growth substance, previously designated as coenzyme R. Synthetic humic acids, prepared by boiling sugar in sulphuric acid with the subsequent addition of iron salts, failed to produce a marked increase in the growth of rhizobia even though the iron in such preparations is essentially as available as that in natural humic acids. There was, likewise, no indication that traces of other elements in natural humic acid played a very important part in the growth stimulation. These findings contrast very definitely with simultaneous observation made with *Azotobacter* which show a marked response to available iron and other elements, especially molybdenum and vanadium, and no response to coenzyme R. The iron requirements of rhizobia are evidently small since additions of available iron to the ordinary synthetic medium frequently produced an almost negligible effect, and rarely was the stimulation greater than 10–25 per cent.

A19. Numbers of Rhizobium in Humus Cultures. ALVIN W. HOFER, New York Agricultural Experiment Station, Geneva.

An investigation to determine numbers of *Rhizobium* in humus cultures was carried out by plate count, and later by inoculation of seeds planted upon Crone's agar in small bottles. Some cultures were purchased on the open market, and others were manufactured from humus submitted by three laboratories. In the latter case, plant counts were carried out over a period of a year. A further study was made by tests of cultures which had passed their date of expiration, to study humus cultures as contrasted to pure cultures of the sand or agar type.

Plate counts were discontinued at the beginning of the investigation, because of the presence in great numbers of a weakly fluorescent organism which produces colonies very similar to those of *Rhizobium*. Inoculation tests showed this organism to be incapable of nodule formation; apparently it occurs in the humus, and is stimulated to growth by addition of the nutrient media containing the nodule organisms. No evidence was discovered to indicate that its presence is harmful to *Rhizobium*.

The results of the tests showed that the number of legume bacteria was extremely variable in the humus cultures tested. Cultures made just previous to the investigation proved to have a higher count after one week than they did when inoculated. After 50 days, the count was usually less than at the time of inoculation, while during the course of a year, it dropped to less than one per cent of the count at inoculation. The results indicated, however, that sufficient numbers of nodule bac-

teria can be added to the humus during manufacture to make up for the drop in numbers, unless a humus unfavorable to *Rhizobium* is used. Although comparisons of inoculants which had passed their date of expiration indicated that sand and agar cultures retain their inoculating ability for much longer periods than humus cultures, results of official legume inoculant tests show that some manufacturers are consistently producing humus cultures of good nodule-forming ability. Apparently, the technique used in manufacture of legume inoculants is of much greater importance than the question of whether the substrate consists of agar, sand or humus.

A20. The Bactericidal Properties of Certain Plant Juices. J. M. SHERMAN AND H. M. HODGE, Cornell University.

It is well known that because of acidity, tannins or the presence of specific toxic substances (such as catechol and protocatechuic acid in pigmented onions) some plant juices have germicidal properties. On the other hand, it is not generally thought that plant juices are endowed with that more subtle type of bacterial inhibiting substances such as is found in animal fluids, though some European workers do believe in the existence of such "humoral" factors. Apparently thought on this subject has been greatly influenced by the negative findings of Russell on the juice of the canna, more than 40 years ago. It should be noted, however, that Russell clearly indicated his belief, almost as a self-evident fact, that the tissues of plants have such an action even though it could not be demonstrated in the expressed juice.

We have demonstrated a relatively mild but distinct bactericidal action in the raw juices pressed from the heads of cabbages and from the roots of turnips. The magnitude of this "germicidal action" is somewhat greater than that found in freshly drawn milk but not nearly so great as that of fresh normal blood. Attempts to demonstrate a similar property in juices pressed from carrots, parsnips and cucumbers failed. The bactericidal substance of cabbage and turnip juice is destroyed at 60°C. for ten minutes and at higher temperatures. The factor in cabbage juice is apparently removed by passage through a Berkefeld N filter but not by the V candle, and is adsorbed by activated carbon. The bactericidal action of these plant juices was demonstrated with *Bacterium coli*, *Bacterium aerogenes*, *Pseudomonas campestre*, and with the natural mixed bacterial flora from the exterior of the plant tissues from which the juices were expressed. The duration of the "germicidal period" is 8 or more hours at 37°C.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CONNECTICUT VALLEY BRANCH

YALE UNIVERSITY, NEW HAVEN, CONNECTICUT, NOVEMBER 16, 1935

AN ANTITUBERCULOSIS SERUM OF HIGH ANTIPOLYSACCHARIDE TITRE. Robert M. Thomas, Department of Pathology, Yale School of Medicine, New Haven, Connecticut.

The serum was produced by the hyperimmunization of a horse with intravenous injections of defatted H-37 tubercle bacilli. It contains a small amount of anti-protein antibody and a large amount of anti-polysaccharide antibody. When injected together with living H-37 tubercle bacilli into the skin of rabbits the resulting lesions are considerably reduced in size and intensity. Absorption of the serum with polysaccharide renders it inactive. No demonstrable passive immunity is conferred by intravenous injection of the serum. The localization of tubercle bacilli injected intravenously in areas of skin previously injected with testicle extract is inhibited if serum be added to the testicle extract. The intrathecal injection of an anti-serum bacillary mixture in rabbits results in a reduction in the number and extent of the tuberculous lesions which develop in the meninges, as compared with the lesions developing in controls given normal horse serum plus bacilli. Four of five such controls became paralyzed. One of the five animals injected with anti-serum developed a transient paralysis. Guinea pigs injected intradermally showed reduction in the size of the skin lesions, but visceral infection was not interfered with. Further experiments are under way.

OXIDATION-REDUCTION POTENTIALS OF CERTAIN ANAEROBIC AND FACULTATIVE ANAEROBIC BACTERIA: I. DIFFERENTIATION OF SPECIES OF THE SPORE-FORMING ANAEROBES BY POTENTIOMETRIC TECHNIQUE. *Russell W. H. Gillespie*, Department of Bacteriology, Yale University, New Haven, Connecticut.

The main object of the present study was to determine whether the various species of *Clostridium* tend to establish final reduction potentials of the same intensity, as was suggested by the observations of Plotz and Geloso, or whether different O/R levels are attained which may be regarded as characteristic for the different species. One may anticipate variation among the members of a closely related group, on the basis that different species exhibit different metabolic activities.

In a study of eight different strains of *Clostridium botulinum* we found that they exhibited only slightly different reduction potentials. The investigation was extended to include other species of *Clostridium*, namely, *Cl. tertium*, *Cl. Welchii*, *Cl. sporogenes*, *Cl. botulinum*, *Cl. tetani*, *Cl. oedematiens*, *Cl. chauvei*, and *Cl. lentoputrescens*.

The potentials of the different species were found to differ to a very appreciable extent both as to final potential levels and differences in trend. Most of our effort has been devoted to the differentiation of different strains of *Cl. botulinum* from those of *Cl. tetani*, on the basis of final reducing intensity established in these cultures under controlled conditions of anaerobiosis and H-ion concentration.[1] The differences observed appear to be significant.

STREPTOCOCCAL ANTIFIBRINOLYSIN TEST IN CLINICAL USE. *L. Furcolow and D. Fousek*, Department of Pediatrics, Yale School of Medicine, New Haven, Connecticut.

Serological diagnostic tests for hemolytic streptococcus infections have been used for some years. The difficulty and expense of the antistreptolysin test of Todd has limited its usefulness. In the present study we have worked with the antifibrinolysin test of Tillett and Garner which depends upon the production by human pathogenic hemolytic streptococci of a substance which dissolves human fibrin clot. If an individual has recently recovered from a hemolytic streptococcus infection his blood clot will withstand the lytic action of the streptococcus.

Eighty-four tests were performed on seventy individuals, and of these twenty-two tests gave negative results. Of the negative tests only six were on cases of suspected streptococcal infection. Twenty-five reactions fell within the borderline group; they required one to three hours to dissolve the clot. Of this number twenty-two were from individuals with proven streptococcal infections or from contacts. Thirty-seven tests were positive, requiring more than three hours to dissolve the fibrin. All of these but one were from proven streptococcal infections, the exception being a case of staphylococcus septicemia.

The conclusion reached was that the fibrinolysin test is an easy and accurate indicator of recent streptococcal infection.

SOME PATHOGENIC MANIFESTATIONS OF CERTAIN OF THE NEISSERIAE. *Frank M. Huntoon*, New Haven, Connecticut.

In certain cases of chronic respiratory disease, notably pulmonary tuberculosis, which are characterized by excessive coughing, members of the *Neisseria* group have been isolated from the lung sputum. These include *Neisseria sicca*, *N. flava*, *N. perflava* and *N. subflava*. Many such cases appear to be allergic to the proteins of the isolated organism, as was shown by intradermal skin tests with antigens prepared from the homologous organism. Desensitization with the antigen is followed by improvement in the symptoms (particularly the cough). Since such organisms can produce an acquired allergy, causing increased pulmonary irritability, it follows that they must be regarded as possible pathogens. Their significance in any particular case must be determined by trial.

THE EFFECT OF CYSTEINE ON TETANUS TOXIN. *Philip B. Cowles*, Department of Immunology, Yale School of Medicine, New Haven, Connecticut.

The fact that neutralized cysteine-HCl will lower the titer of both diphtheria and tetanus toxins, as mentioned by Locke and Main (1931), has been

[1] pH was controlled with glass electrodes accurate to within 0.02 to 0.03 unit pH.

confirmed. With tetanus toxin, in particular, the effect is very marked. Cysteine-toxin mixtures containing per cubic centimeter 1.0 mgm. of cysteine and 1500 M.L.D. of toxin (white mice) or 5.0 mgm. of the former and 25,000 M.L.D. of the latter are detoxified in 24 and 48 hours, respectively. The reaction occurs in air, but not *in vacuo*, and it will not take place in serum or in defibrinated blood. That the toxin is changed to toxoid is shown by its ability to combine with antitoxin and to stimulate antitoxin formation in the rabbit.

THE CENTRAL NEW YORK STATE BRANCH

THIRTIETH MEETING, GENEVA, NEW YORK, NOVEMBER 23, 1935

MACROSCOPIC COLONY DEVELOPMENT ON PLAQUES OF SOIL FROM SAMPLES TAKEN AT VARIOUS DEPTHS. *H. J. Webb*, Department of Agronomy, Cornell University, Ithaca, New York.

DETECTION OF RECONTAMINATION OF PASTEURIZED MILK BY LABORATORY METHODS. *W. H. Chilson and M. W. Yale*, New York State Agricultural Experiment Station, Geneva, New York.

Samples were taken monthly for one year at two pasteurizing plants. Counts of organisms of the colon group were made on Leifson's lactose desoxycholate agar. Of 292 samples examined, 229 were pasteurized. Colon organisms were absent from 1 cc. quantities of freshly pasteurized milk at both plants, but were present in the first milk from the bottler at one plant in 8 instances out of 12, in numbers ranging between 1 and 3,000 per cubic centimeter. Their presence indicated recontamination in four monthly tests where it was not shown by standard plate counts. In one series where colon organisms were not found, standard plate counts indicated recontamination.

A study of an additional 108 samples indicated that recontamination was due to: (1), improper sterilization of the milk pump; (2), failure due to steam outlet valves containing a slight amount of raw milk leaking from holders; and (3), condensation water and milk, containing between 1,200 and 5,000 colon organisms per cubic centimeter, which dripped into the bottle from a coupling frequently adjusted by the hands of the men at the bottler. When these conditions were corrected, colon organisms were not recovered from 1 cc. quantities of the samples of pasteurized milk.

THE SIGNIFICANCE OF LOW AGGLUTINATION TITRES IN BANG'S DISEASE. *H. S. Cameron and H. L. Gilman*, New York State Veterinary College, Cornell University, Ithaca, New York.

HYDROGEN-ION CONCENTRATED TUBERCULOPROTEIN ANTIGEN. *E. B. Hanan and Sophia Zurett*, Buffalo City Hospital and University of Buffalo Medical School, Buffalo, New York.

The tuberculoprotein is prepared from an attenuated H37 strain of the tubercle bacillus grown for six weeks on glycerin broth. The organisms are filtered, washed free of glycerin and dried *in vacuo*.

One gram of the dried bacilli is heat extracted under a reflux condenser with 100 cc. of buffer solution at pH

7.0. Following the separate extraction of each of three grams of the organisms, the extracts are centrifuged and the protein is precipitated by addition of the supernatant fluids to buffer solutions adjusted to pH 2.8. The precipitates are washed with fresh buffer solution (pH 2.8) until the supernatant fluids are colorless.

The precipitates are then concentrated and dissolved in 100 cc. of pH 7.0 buffer solution and dialyzed against neutral distilled water for from 5 to 8 days. The dialyzed solution is placed in a sterile bottle and, then, heated at 65°C. for thirty minutes. Then, 0.5 per cent phenol is added and the antigen is ready for standardization and use in the complement fixation test for tuberculosis.

The antigen gives fixation of complement in 80 per cent of known cases of tuberculosis. Its chief advantage appears to be that it does not give "false positives" with syphilitic sera.

SOME ACID-FAST ACTINOMYCES FROM SOIL. *Ruth Gorden*, New York State Veterinary College, Cornell University, Ithaca, New York.

CULTIVATION IN VITRO OF THE VIRUS OF HUMAN ENCEPHALITIS, ST. LOUIS TYPE. *J. T. Syverton and G. P. Berry*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

Evidence is presented of the successful *in vitro* propagation of the virus of human encephalitis, St. Louis type, "Daily" strain, in the presence of living cells. Two similar series of experiments are reported. The source of virus was the brain tissue of mice, dead or moribund following intranasal instillation of the virus. Virus in the bacteria-free supernatant fluid of a centrifuged 10-per-cent suspension in broth, lethal for mice in a dilution of 10^{-4}, was used in 0.3 cc. amounts to initiate the cultures. This was added to 2.7 cc. of culture medium, consisting of 1 or 2 drops of finely minced mouse embryonic tissue suspended in a mixture of 1 part of rabbit serum to 2 parts of Tyrode's solution, contained in a 50 cc. Erlenmeyer flask. The inoculated flasks were incubated at 37°C. with successive passages made every fourth day by direct transfer of 0.3 cc. of the preceding passage flask to flasks of similarly prepared medium. In each series of experiments the virus was carried through 19 transfers. The presence of virus in each bacteria-free culture generation was confirmed by testing on mice. Experiments on the serological specificity of the culture virus and its immunological relationships following repeated cultivation will be reported shortly.

IN WHICH CROSS-INOCULATION GROUP DO CERTAIN UNCLASSIFIED LEGUMES BELONG? *J. K. Wilson*, Department of Agronomy, Cornell University, Ithaca, New York.

VACCINATION OF CALVES AGAINST JOHNE'S DISEASE. *W. A. Hagan*, New York State Veterinary College, Cornell University, Ithaca, New York.

THE MAXIMUM TEMPERATURE OF INCUBATION PERMITTING GROWTH OF BETA-HEMOLYTIC PATHOGENIC STREPTOCOCCI. *Marion Snyder Reed*. New York State Agricultural Experiment Station, Geneva, New York.

A study of the upper limits of growth of 92 authentic strains of beta-hemolytic streptococci was undertaken.

Young cultures were inoculated into veal infusion broth containing 1 per

cent glucose and 0.004 per cent brom thymol blue (pH 7.4). The media were brought to the desired temperature in a water bath prior to inoculation and, then, immediately returned to the water bath. Rubber caps were used over the tubes to prevent rapid evaporation, with the resulting cooling effect. Constant temperature was maintained by submerging the tubes in a beaker of water during the incubation. Average temperatures were obtained from readings taken three times a day. Sufficient change in hydrogen ion concentration to effect the color of brom thymol blue, was used as an index of growth.

A majority of the beta-hemolytic pathogenic streptococci failed to grow at temperatures above 41.2°C. In no case was growth observed above 43.9°C. in 88 strains of this type. A limited number of non-pathogenic beta-hemolytic streptococci grew at temperatures as high as 49.5°C.

A Study of the Effect of Compounds Containing Zinc on the Activities of Certain Soil Microörganisms. *W. L. Lott*, Department of Agronomy, Cornell University, Ithaca, New York.

Studies on the Relationship of the Viruses of Rabbit Fibroma (Shope) and Infectious Myxomatosis (Sanarelli). *G. P. Berry, J. A. Lichty, and Helen M. Dedrick*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

Shope's findings, reported in 1932, that the viruses of Rabbit Fibroma and Infectious Myxomatosis are immunologically related, have been confirmed and extended. Infection with either fibroma or myxoma virus induces a resistant state against either virus, this state being accompanied by neutralizing properties against either virus. These results followed the development of a sensitive neutralization technique, and the establishment of Myxomatosis in the wild cottontail (Genus, *Sylvilagus*) rabbit. In this new host Myxomatosis is seldom fatal.

We investigated this relationship by an approach suggested by Griffith's studies in 1928 on the transformation of pneumococcal types. Domestic rabbits were inoculated with a mixture of fibroma virus and heat-inactivated myxoma virus. The inoculated rabbits developed characteristic Myxomatosis with typical histopathology. Secondary lesions yielded myxoma virus on transfer, and normal rabbits were infected by simple contact. Controls have been adequate and have included several different strains of fibroma and myxoma virus. We believe that we have changed fibroma virus into myxoma virus. We suggest that this type of approach may prove useful in exploring other virus relationships, for instance, in the animal pox group, in the foot and mouth disease group, etc.

Certain Modifications of Standard Methods for the Bacteriological Analysis of Milk.

1. Temperature of Incubation. *C. S. Pederson and M. W. Yale*, New York State Agricultural Experiment Station, Geneva, New York.
2. Media. *G. J. Hucker and C. S. Bowers*, New York State Agricultural Experiment Station, Geneva, New York.

Cultural Characteristics of Aerobic Gram-Negative Bacilli Isolated from Genito-Urinary

INFECTIONS. *L. A. Sandholzer*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

A study of 530 cultures of aerobic Gram-negative bacilli, isolated from 283 patients suffering from urinary infections, has been made. The organisms have been subjected to detailed investigation to permit exact taxonomic allocation with the aim of ascertaining whether or not any relation between the species or types and the clinical course of the infections exists. This paper deals only with the bacteriological findings.

The cultures fell into 6 genera; *Escherichia* 83 per cent; *Aerobacter* 13 per cent, the remaining 4 per cent being distributed among *Salmonella*, *Proteus*, *Shigella*, and *Pseudomonas*.

Hemolytic activity was determined on rabbit-blood pork-infusion agar in poured plates. All of Brown's types of hemolysis were observed, but no correlation between the type of hemolysis and the taxonomic position was apparent.

A STUDY OF THE MICROFLORA OF ERODING SOILS AND OF THE RUN-OFF FROM THESE SOILS. *J. H. Schubert*, Department of Agronomy, Cornell University, Ithaca, New York.

METHODS OF PROTECTING PUBLIC MILK SUPPLIES AGAINST THE INCLUSION OF EVIDENTLY ABNORMAL MILK. (Read by title.) *Robert S. Breed and Richard Eglinton*, New York State Agricultural Experiment Station, Geneva, New York.

Recently it has been suggested that the exclusion of apparently abnormal milk from public milk supplies can be at least partially accomplished by using a "strainer-dipper" on cans of milk as received at milk stations. The same purpose may be accomplished by the more time-consuming, but more accurate method of examining samples of milk from each quarter of the udder of the cows in a given area.

Out of 836 cows in the Geneva, New York region from which samples were taken in a preliminary survey, 46 had quarters with sufficiently active bacterial infections to cause the secretion of abnormal milk. In some cases more than one quarter was infected so that the total number of quarters affected was 57. The dairymen milking these cows knew that 21 of these quarters were secreting abnormal milk and this milk was kept out of the city milk supply. The milk from the remaining quarters was included up to the time that its existence was detected during this survey. In a surprising number of cases the abnormal milk had developed as the result of an injury. Additional surveys are being made to determine the practicability of such intensive routine control methods.

NORTHERN CALIFORNIA-HAWAIIAN BRANCH

OCTOBER 30, 1935

A TECHNIC FOR THE ESTIMATION OF THE ANTISTAPHYLOLYSIN CONTENT OF HUMAN SERA. *Lucien D. Hertert*, Hooper Foundation, and *John W. Cline*, Stanford University.

A study concerning the antihemolytic factors against the hemotoxin of *Staphylococcus aureus* and a routine titration of human serum.

The most satisfactory technic for standardizing the staphylolysin utilized toxin diluted with 10 per cent

broth-saline carried in 1.0 cc. of solution, against 1.0 cc. of a 2 per cent suspension of rabbit erythrocytes. A primary incubation of one hour at 37°C. in a water bath followed by 16 hours in the icebox gave clear-cut reactions.

Neutralization was accomplished using 0.05 cc. of serum heated for 30 minutes at 55°C., 0.1 cc. of toxin in ascending increases of 0.1 cc. per tube (each 0.1 cc. contained one hemolytic unit). After a one hour fixation in the icebox, 1.0 cc. of the erythrocytes was added, tubes shaken, and incubated for one hour in a 37°C. water-bath, followed by 16 hours in the icebox. Each tube showing complete inhibition is the equivalent of 20 antihemolytic units per cc. of serum. Controls were included covering a standard serum, hemolytic activity of the toxin and erythrocyte stability.

The normal values range from 20 to about 100 antihemolytic units. Very high titers are encountered following acute staphylococcal infections although chronic infections generally show titers of less than 20 units. Patients with tuberculosis and convalescing from acute osteomyelitis of staphylococcal origin show the highest titers, those with recurrent furunculosis the lowest.

RAT-BITE FEVER FROM FIELD MOUSE. *R. J. Reitzel, Arthur Haim and Kirk Prindle*, University of California, School of Medicine.

A boy, aged 14, was bitten by a wild field mouse and developed rat-bite fever. Venous blood and emulsified lymph node obtained at biopsy and injected into white mice revealed *Spirillum minus*. Cure was effected by the administration of neo-arsphenamin.

THE ANTIGENIC AND PATHOGENIC CHARACTERISTICS OF BRUCELLA ABORTUS, UNITED STATES BUREAU OF ANIMAL INDUSTRY STRAIN NINETEEN. *C. M. Haring and J. Traum*, University of California.

In experiments, supported in part by the United States Bureau of Animal Industry, 310 cattle have been subjected to subcutaneous injections with a strain of "reduced virulence" used by commercial laboratories in preparing live *Brucella abortus* vaccine for use on cattle. Dosages ranged from 75 to 300 billions of living organisms.

The results of trials on four of the vaccinated animals and an equal number of controls support the conclusion of Cotton and Buck that one subcutaneous injection after sexual maturity confers immunity to fetal and placental infection for at least two years. Immunity was also conferred to bacteremia and shedder conditions in the milk.

As yet, sufficient data are not available to confirm definitely the conclusion of Cotton and Buck that calves vaccinated before sexual maturity with culture 19 retain resistance to *Brucella* after they have become pregnant.

Association of cattle vaccinated while nonpregnant with healthy nonvaccinated cows has not resulted in any evident spread of Brucella infection.

Passage through guinea pigs, goats and cattle has failed to change the virulence and cultural characteristics of the strain.

STUDIES ON EXPERIMENTAL PULMONARY ABSCESS. I. TRANSMISSION OF THE DISEASE TO MONKEYS BY THE BRONCHIAL ROUTE. *Charles Weiss, and Marian C. Shevky*, Mount Zion Hospital, San Francisco, and Divi-

sion of Thoracic Surgery of the University of California.

A method is described for the production of pulmonary abscess in Java monkeys (*Macacus irus*). The infectious material was obtained at operation from the lung of a patient with chronic pulmonary abscess and the anaerobes which it contained (*Bacterium melaninogenicum*, spirochaetes, various types of fusiforms, anaerobic streptococci, etc.) were kept alive by frequent passage through the inguinal tissues of guinea pigs, as suggested by Kline and Smith. With the aid of a fluoroscope and a radio-opaque ureteral catheter, the inoculum (pus removed from the inguinal lesions of the guinea pigs), was introduced directly into a lower lobe bronchus, the bronchus being obstructed in order to prevent expulsion of the bacteria during cough.

Of eight animals thus injected, three developed large, putrid abscesses; one, pneumonia with several small abscesses, and a fifth, pneumonia; while three others remained essentially normal. Control experiments with heated pus and agar produced only transient atelectasis, without evidence of pulmonary suppuration.

The evidence presented suggests that pulmonary abscess may be due in certain cases to aspiration into the lungs of infectious material derived from the upper respiratory passages and the plugging of the corresponding bronchus.

LATENT INFECTIONS[1]

K. F. MEYER

From the George Williams Hooper Foundation, University of California, San Francisco, California

In the contemporary literature which deals with the infectious diseases, the designation "symptomless infection" is used with increasing frequency. The question immediately arises: Do recent observations, experimental and clinical, and new epidemiologic experiences justify the creation of this term? Is it not likely that a new word has been coined to describe the well-known state of "latent infections"? No one will deny that the usual signs of an infectious malady—the manifest and latent stages—may be readily bridged by the so called rudimentary or "abortive" forms of the disease. In other words, the symptomatology of an infection oscillates between the frank, clinically typical disease and the unrecognizable reaction. The persistence of a disease agent from the time of its entry into a host or the "symptomless" state of infection, which may follow clinical recovery, has been described, since the discovery of the tubercle bacillus by R. Koch in the tissues of apparently healthy individuals, as a "latent infection." Furthermore, one was satisfied to characterize certain atypical forms of a disease as "abortive" when it was believed that the disease-producing virus multiplied only to a moderate extent and consequently induced feeble reactions or no symptoms at all. The recognition of the "abortive" cases has gradually led to a broader conception of the infectious maladies. Since a disease is generally considered from an etiologic point of view, it is not surprising to note the descriptions of scarlet fever without a rash and poliomyelitis without paralysis. Whether or not the variant types of a disease are truly "abortive" forms has certainly not been determined for a great many infections. In fact, the

[1] Presidential address prepared for delivery before the Society of American Bacteriologists at its thirty-seventh annual meeting, New York, December 27, 1935.

recent intriguing studies by Paul, Salinger and Trask and others amply illustrate the difficulties which may be encountered in the interpretation of the minor illnesses which are observed in association with clinical poliomyelitis. Moreover, it must be reserved for future experimental studies to furnish proof that the attenuated clinical course of an infection, which cannot be recognized without the most searching medical examination, is truly the result of a supposedly diminished multiplication of the disease agent. Observations to be discussed later indicate that a number of additional factors are of equal if not of greater importance.

What is the proper concept of a "symptomless infection?" An observation made by Charles Nicolle in 1912 in connection with certain studies on typhus lead to its precise definition by Nicolle and Lebailly in 1919. These investigators had noted that, occasionally, in a series of guinea pigs experimentally infected with typhus blood, one or several would fail to show a characteristic febrile reaction. The blood or organs of such animals transferred to other guinea pigs would, however, induce the typical fever and thus prove the existence of the virus in the animals which had shown no symptoms. This peculiar behavior probably conditioned by a particular constitutional state of the guinea pig was designated by Nicolle as "une infection inapparente ou silencieuse." In recent years, this phenomenon has been intensively studied and amply confirmed by numerous investigators (Doerr, Breinl and others). Thus, Breinl showed that the simultaneous but separate injection of virus and serum in proper dosage produces an afebrile infection with regularity. Under the influence of the immune serum the virus multiplies and reaches the same concentration in the blood as in the non-treated animals with the only difference that the onset of the virus propagation may be delayed for a few days. The virus growth curve, except for a slight shift in time, is the same in both animals, irrespective of the fact that the serum-treated guinea pigs remain permanently afebrile. That this type of infection leaves a permanent immunity was anticipated and experimentally proven. A further analysis of these interesting observations by Breinl and Singer demonstrated that not only the fever but the

other typical symptoms such as the perivascular infiltrations, the blood monocytosis, and the loss in weight were equally absent. Only the polymorphonuclear leucocytes and the lymphocytes were slightly but temporarily increased for a period of time which ordinarily corresponds to the febrile state of the typically reacting animal. Obviously, this peculiar type of infection is not absolutely "symptomless" but it deviates so markedly from what is generally recognized as the norm that the formulation of a new term appeared justified. The deciding criterion of the "symptomless infection" is a rise and fall of the virus growth curve. Under normal conditions this proliferation of the virus induces the well-known symptoms. However, under certain conditions—individual disposition of the animal (Nicolle), influence of immune serum (Weil and Breinl), infection with louse-passage virus (Breinl) or shifts in the electrolytes of the body (Breinl and John)—the symptom complex fails to materialize though the typical virus multiplication curve remains unchanged. The parasite increases in the macroorganism to the characteristic saturation point (10^{-5} M.L.D. in the brain) without provoking a noticeable defense reaction, other than a completely "symptomless" process of immunization. This phenomenon should be considered for the time being as a condition decidedly different from that which is generally described as "latent infection." The term "symptomless" or "inapparente infection" should be applied only to those maladies in which the virus is distributed with respect to location, time and quantity in a manner characteristic for the usual course without, however, causing the appearance of clinical or anatomical signs of disease. As late as 1933 Nicolle declares himself opposed to the recognition of this type of infection as a "latent process." He defines a "latent infection" as a subacute or chronic state of disease without evident symptoms in which the carrier harbors the disease agent. The microbe may have been acquired through a preceding disease; it may regain its infectiousness and thus affect the carrier or may serve as a source of an infection to other individuals.

Nicolle warns against the error of describing a disease as "inapparente" should the pathogenic agent escape detection by the

examiner. Non-visibility is not synonymous with "inapparence." Finally, the exponent of the term "inapparente" vigorously demands that it be limited to the infectious disease, but not to deficiencies or dystrophies as already proposed by Moriquand, Velu and Balozet.

True "symptomless infections" may be expected to develop when agents similar to the spotted fever virus are involved. According to Nicolle,[2] this type of infection is observed in the following diseases and species of animals:

Epidemic European spotted fever in man, rats and mice
Endemic rat typhus in guinea pigs, monkeys, mice and rats
Kala-azar in the spermophiles (*Citellus citellus*) and in dogs
Weil's disease in rodents and guinea pigs
Treponema pallidum infections in mice, rabbits, possibly man
Variola in the rabbit, dog and cat
Yellow fever in man and monkeys
Poliomyelitis
Herpes and encephalitis in man
Lymphogranulomatosis in the monkey
Malaria in man and probably in *Anopheles*
Spirochaeta recurrentis in the gondi and other animals (*Ctenodactylus gondi*)
Infectious anemia of horses, rodents and ticks
Dourine
Fowl plague
Brucella infections, not infrequently in man, constantly in goats

This brief compilation merely suggests that the world may be flooded by a sea of disease agents which, as a rule, produce merely a "symptomless infection." In order to emphasize the significance of the problems involved, additional examples are herewith presented.

In 1929 Blanc, Caminopetros and Manoussakis injected a human being intravenously with 3.5 cc. of serum collected from a patient with dengue fever. He remained clinically well but the blood removed from him on the third day after the administration

[2] Destin des Maladies Infectieuses, Paris, Felix Alcan, 1934, p. 119; Introduction à la carrière de la médecine expérimentale, Paris, 1932, pp. 85–91.

of the serum and reinoculated in an amount of 7 cc. into a third person proved to be infectious. A typical dengue infection was produced. Subsequently, the person with the "inapparente infection" was found to possess a specific immunity towards the dengue virus. Nine cubic centimeters of whole virulent blood, which proved virulent in a control experiment induced neither manifest symptoms nor did the blood of the person reacquire infectious properties. The same investigators found in 2 out of 6 experiments that guinea pigs injected with dengue blood may yield no febrile reactions while their blood transferred to human beings may prove infectious. Transfers to guinea pigs were not successful, doubtless on account of the very marked variability of their individual susceptibility. Similarly, experiments conducted by Blanc, Caminopetros, Dumas and Saenz on monkeys (*Macacus cynomolgus* and *Cercopithecus callitrichus*) have shown that these species of animals pass through "inapparente infections" when injected with virulent dengue blood. They are neither visibly ill nor is their temperature elevated. However, their blood, avirulent 24 hours after the injection, becomes infectious for man on the 5th, remains so until the 8th but loses its pathogenicity on the 12th day. It is of interest that the infectiousness can be proven only by transfer experiments to man. Reinoculations on species of monkeys that react quite regularly with an "inapparente infection" to an injection of dengue blood invariably failed. These observations, provided they are confirmed, would indicate that the extinction of an "inapparente infection" in its first generation in certain animal species may be a phenomenon of greatest general biologic significance.

Under the influence of a potent immune serum, the course of a cattle plague or hog cholera infection may be so modified that "symptomless infections" are by no means infrequent. Doubtless, the combined influence of an inherent natural resistance enhanced by the immune serum is responsible for these results. That the virus increases in the hog, which has been treated with serum and then injected with virulent blood, may be readily proven by systematic transfer inoculations to susceptible young pigs. As an aftermath of this infection, which passes through an

afebrile course, a permanent active immunity is produced. Aside from the "inapparente infection," a hog cholera virus invasion may be followed by a typical "latent infection." The virus may persist for 3 weeks in the blood and up to 103 days in the lymph-nodes (McBryde, David); it may even be discharged irregularly in the urine for a much longer time.

A measles infection under the influence of convalescent serum may be modified to an unrecognizable condition, absence of fever or Koplik's spots with an indefinite rise in temperature. Yet, it is well-known that these so called "mitigated forms" of measles are highly infectious and not infrequently the nodal centers for large epidemics.

In connection with the studies on herpes occasional "inapparente infections" have been noted. Levaditi has produced in the rabbit encephalitides without symptoms; their existence was placed in evidence after death through the demonstration of the neuropathologic changes. Other workers have demonstrated the virus in considerable quantity in the nervous system, irrespective of the fact that the clinical signs of the brain infection were rudimentary or entirely absent. Olitzky and Long noted virus propagation in the brain of guinea pigs without encephalitis and Remlinger and Lebailly found the herpes virus "non-pathogenic" for the turtle (*Testudo mauretanica*) although the infective agent was demonstrable for a long time by transfer of the brain tissue into the susceptible rabbit. Virus increase in the central nervous system and the pathogenic effect are apparently two entirely different processes. In fact, no one is in a position to-day to explain the reason why an increase in the virus is followed in one instance by an acute fatal disease while manifesting itself in another experiment as a "symptomless infection."

It is the merit of the late Professor Kolle to have applied the term "symptomless infection" to certain observations which he and his associates, Evers and Prigge, had made in connection with the experimental study of syphilis. Rabbits treated with bismuth preparations and subsequently injected with syphilitic material are apparently protected against a luetic infection. Although a chancre formation and the enlargement of the lymph-

nodes—the usual criteria of a "take"—are missing, the spirochaetes remain viable without producing lesions. Since it had been shown by Pearce and Brown that the popliteal lymph-nodes of rabbits recovered from a syphilitic infection retain the spirochaetes, it occurred to Kolle and Evers to extirpate various nodes of the bismuth-treated and clinically unaffected animals, and transfer them into the testis of normal rabbits. In numerous instances typical chancres were produced. Thus, it was shown that the bismuth preparation failed to sterilize and although it prevented the development of local and general syphilitic lesions, it permitted the general distribution of the spirochaetes. Since this phenomenon was discovered under more or less artificial conditions, it was at first believed to be of little or no general significance. However, within a short time the field of "inapparente" spirochaetal infection was broadened. It was noted that rats and mice may be readily infected by placing pieces of gummatous tissues under the skin of the back or the testes. The local lesions healed without the formation of a chancre. After a lapse of many months the subinoculation of the lymph-nodes, spleen or brain of the infected muridae produced in rabbits typical chancres. Furthermore, the transfer of organ particles removed from mice with "inapparente infections" again created in this species a "symptomless infection." The mice became hosts for the *Treponema pallidum* without showing clinical or anatomical manifestations. Comparative studies on rabbits leave no doubt that the distribution and the degree of the spirochaetal increase within the tissues correspond in every respect with that established for the manifest infections. Inflammatory reactions are not histologically demonstrable in the animals which are not visibly sick. For the latter the designation "residual brain infection" and for the former "symptomless infection" would be correct.

In many ways analogous, but not identical, are the observations dealing with the *Spirochaeta recurrentis* infections of burrowing rodents and other animals. Experiments carried out in California indicate that chipmunks and tamarack squirrels may harbor spirochaetes in their tissues. Particularly late in the fall

these findings are by no means infrequent. Since anatomical lesions are invariably absent and the presence of the spirochaetes is established by subinoculation into mice, the majority opinion of investigators is that these rodent infections are "symptomless." A few carefully controlled tests, however, have shown that this chronic state is preceded by an acute infection which has conferred a certain degree of immunity on these carriers. These rodents serve as a reservoir for the spirochaete and continue to furnish the parasites which, through the agency of *Ornithodorus Hermsi*, a newly discovered vector are accidentally transmitted to man. With this incomplete evidence at hand one would prefer to designate the process in the rodents as a "latent," though in some other animal species and the tick as an "inapparente," or according to Nicolle as an "invisible" infection.

These and similar reflections now logically demand an analysis of the term "latent microbismus," "silent or resting infection" introduced during the war by surgeons to characterize the delayed appearance of tetanus and gas gangrene infections. It is well-known through the animal experiments of Vaillard and Rouget (1892), Tarozzi (1906), Canfora (1908) and Koser and McClelland (1918) that spores of the tetanus bacillus may remain at the site of the inoculation or may be carried by phagocytosis to various parts of the animal body where they persist for long periods. Although it is as yet unknown whether such persisting spores are free in the tissues or inclosed in phagocytes, the fact remains that they may be activated by various chemicals, bacterial products, etc. and thus induce typical tetanus. For example, Schneider demonstrated tetanus spores in the liver, spleen and even the blood of rabbits 43 days after their injection with detoxified spores. Every process, which results in tissue necrosis, spontaneous bacterial infections or artificially induced disease, may lead to an attack of tetanus in these animals which harbor the spores. This peculiar "latency" has been experimentally established for many other toxicogenic anaerobes. In fact, it is well-known that anaerobic spores introduced into the human body by traumatic injuries and gunshot wounds, especially shrapnel splinters, may remain within the wound or scar tissue without

causing the least reaction. These spores, whether deposited with or without a foreign body, are a constant source of danger as numerous observations collected between 1914 and 1919 have definitely shown. An aseptic operation as, for example, the removal of shot fragments performed weeks, months or years after the original injury may lead either to a local or general tetanus (Courtois, Suffit and Giroux) or a rapidly fatal gas gangrene infection (Penhallow, 1916, Lesne, 1916, Phocas, 1915, Plant and Roedelius, 1918). There is no doubt that an individual, who, in the interval between the injury and the subsequent operations, carries the seeds of tetanus or gangrene, is in a state of "latent infection." However, it is equally obvious that these forms are by no means comparable with the classical "symptomless infection" as illustrated by certain *Rickettsia* and virus infections. What are the differences? A multiplication of the microbe in the scar may with considerable certainty be excluded. Furthermore, this lack of vegetation of the spores is not due to any active immunological effort on the part of the host, but is probably conditioned by factors which influence the growth phases of the microörganism. What are the factors which temporarily inhibit and later activate the vegetation of disease agents? Through a series of brilliantly conceived experiments Fildes (1927) came to the conclusion that the normal positive oxygen tension of the healthy tissues is sufficient to account for the lack of germination. This may be reduced in various ways: A slight inflammatory process may cause through capillary thrombosis a hindrance of oxygen diffusion or the leucocytes may place an increased demand on the oxygen; perhaps the presence of foreign particles induces local asphyxia and favorable respiratory requirements for the germination of the spores. There is no regulated incubation, generalization through the blood stream, no critical disappearance of the disease agent and no subsequent immunity. It is not the host *per se* but certain environmental factors influencing the vegetative phases of the microbe which are of importance. Although Fildes' experiments offer a reasonable explanation for an understanding of the factors which initiate the germination of the spores, they fail to elucidate the primary inhibition after

they have gained entrance to the tissues. Whether phagocytosis (Vaillard and collaborators) or the fortuitous absence of contaminating microörganisms of the *Clostridium Welchii* or *Clostridium sporogenes* varieties may have something to do with this apparent inhibition deserves further experimental scrutiny. How far the so-called dormancy of spores as observed in the test-tube has a bearing on this problem has not as yet been investigated. In any event it is advisable to define this intriguing state of nonreactive persistence of anaerobic spores in the tissues of man and animals as "silent or resting microbismus."

Promptly the question arises: Are the well-known diseases with "long incubation times" as, for example, tuberculosis, *Brucella* infections, leprosy, fungoidal disease, processes due to cocci, perhaps rabies, psittacosis, etc., analogous to the conditions just reviewed? In no instance has an absolute or complete inhibition of the growth activities of the implanted microörganism been demonstrated. Moreover, systematic investigations, as far as they have been carried out, have always revealed a definite though occult primary focus. It is supposed that within this focus, growth of the disease agent may be very slight and frequently retarded or completely arrested by factors which are as shrouded in mystery as the primary inhibitory influences on the spores. The experimental studies by Rous and Jones on the protection of bacterial cells from the action of destructive substances suggest one of the factors which may be operative. In any event, all the evidence available marks these conditions as "quiescent infections" in contradistinction from the "silent microbismus."

It has already been mentioned that Nicolle presented sufficient evidence to exclude from consideration as "symptomless infections" those conditions in which the persistence of pathogenic microörganisms on the surfaces of the mucous membrane or even in the blood has been recognized. This differentiation has not been followed. In fact, under the influence of the newer trends to analyze medical bacteriologic phenomena from a general biologic point of view, an interesting terminology has appeared. The word saprophytism is suddenly replaced by *symbioses* (Rim-

pau). Kolle and Prigge in their monograph on "symptomless infection" list as "symbionts" (a misnomer for the Greek word "symbiote" = companion or partner) of the mucous membranes or the blood the following disease agents and their hosts.

Man. *Lamblia intestinalis*, pneumococci, anaerobic spore-bearing bacteria such as *Clostridium tetani* and *Clostridium Welchii*; diphtheria, influenza and Ducrey bacillus, fungi and various species of filaria.

Mammals. *Pasteurella pestis* in rats, fowl chlora bacillus in chickens, various spirochaetes in rodents; *Trypanosoma Lewisi* in rats; *Brucella melitensis* in goats.

Insects. Plasmodia in the *Anopheles*; trypanosomes in *Glossina*, yellow fever virus in *Aëdes Aegypti*, pappataci virus in the *Phlebotomus* flies; spotted fever *Rickettsia* in lice; Rocky Mountain spotted fever in the tick, tsutsugamushi virus in mites, etc.

The list could be readily enlarged; one could include the Bartonelloses of rats, the anaplasmoses and gonderioses, the finding of plague bacilli in the inguinal glands of natives at Dakar by Leger and Baury, etc.

On the other hand, certain corrections and limitations must be pointed out. The *Brucella* infections in goats, particularly the elimination of the organism in the urine or its presence in the milk ducts, is always associated with definite anatomical lesions and is certainly not a state comparable with true symbiosis. Many years ago Carter had shown that the massive ingestion of gametocytes by the *Anopheles* may lead to profound disturbances, even death of the mosquito. The flea tolerates an invasion with the *Rickettsia* of endemic typhus while the louse succumbs (Nicolle). More important in this connection, however, is the pertinent inquiry as to whether such a terminology as "symbionts" and its implication are truly justified.

The presence of a disease incitant on a mucous membrane merely attests that the microörganism has found conditions suitable for its vegetative development. However, it may lack the ability to penetrate and thus be transformed from a potential to a true disease-producing agent. For the infections without symptoms it has been proven that the agent has the power to invade, and that the immunity mechanism of the subclinically

infected macroörganism neither neutralizes its action, if any, nor destroys it more readily than in the visibly diseased. Already Vaughn recognized such a state of affairs when he applied the term *syssitic* to the organisms living commensally on the surfaces of the mucous membranes without production of evident disease. Topley believes that they possess inherently the power of multiplication on mucous membranes or so called *supragliscence*. Under these circumstances, it is quite reasonable to assume that the microörganism has not as yet adapted itself to the macroörganism. The residence on the membranes or in the blood is merely the first act, the beginning of an interplay which may lead from the invisible to the visible disease form.

In the light of modern biologic thinking which gradually permeates the utilitarian concepts of medical bacteriology, it is believed, although by no means proven, that the infection without symptoms is the sequel and not its precursor. Such reasoning outlines the problems still to be solved. By taking into consideration the newer knowledge on symbiosis, one may perhaps define the paths along which future inquiries may be directed. Furthermore, a discussion of this important and significant relationship between various microörganisms and their hosts may in part help to clarify such unfamiliar words as *Epi-* and *Endosymbiosis* (Rimpau) as applied to the microbic behavior under consideration.

During my early training as a zoologist, the phylogenesis of parasitism was always presented as an evolution of the parasitic from non-parasitic types and it was assumed that the pathogenic ascended from the non-pathogenic. With increasing insight it became clear that the reverse may also be true. Benign parasites evolvè from harmful ones. Although it is obviously impossible to observe this phylogenesis, a brief insight into certain families of *Diptera* connected with myiasis offers many convincing observations relative to the correctness of the deductions (Martini). The mode of nutrition of certain species clearly sketches the evolution from true to facultative parasites. The larvae of muscae are rarely found in suppurative wounds, while those of the *Fannia* group may live on wound secretions, although they prefer decomposing plant material. Representatives of the

sarcophaginae generally found in feces and dead flesh may invade wounds and destroy even healthy tissues. The genus *Wohlfahrtia* causes severe destruction in the healthy tissues of man and animals which may be the victim of these flies. *Lucilia caesar* and *Calliphora vomitoria* now used to cleanse wounds (Baer and others) unquestionably destroy the dying tissue fragments. *Chrysomyia* may extend its activities from wounds and pus cavities to healthy tissues. In fact, in Australia a great many Calliphorae deposit their larvae in the wool of sheep. They not only live on the wool but they attack the skin and cause extensive destruction. The larvae of *Cordylobia*, which bore into the skin of dogs and occasionally of man in Senegambia, are primary parasites. They form the transition stage to the obligatory parasites belonging to the subfamilies of *Cuterebrinae* and *Hypoderminae*. Their advanced stage of parasitism is indicated by a retrograde formation of the mouth parts, and it is interesting that many of these forms are living at the expense of the wound secretions which accumulate within the boil. They avoid invasive feeding in the healthy tissues. Polyhagism of certain species of *Dermatobia* is replaced by a specific adaptation of the genus *Hypoderma* for certain ungulatae. *It appears that the gradual and more intimate adaptation to a host and to its mode of living more or less improves the existence of the parasitic species. Simultaneously with this adaptation, the ability to invade other hosts is progressively lost.*

Quite generally, it seems that the less violent action of a parasite on its vertebrate host, the monophagic tendencies and the difficulty of growing on artificial media are signs of a progressive and phylogenetically old parasitism. Viewed from this angle, it is obvious that the slowly fatal or chronic illness of the host is of greater advantage to the parasite than the rapidly deadly or rapidly cured malady. The highest type of adaptation is recognized as a life-long infection of the host (herpes-virus) and, in order to insure persistence of the parasite, a more or less fully developed pseudoheredity. The final stage is reached with the state of symbiosis in which the host becomes infected early during its development and receives no injury through the presence of

the "parasite." In fact, the physiology or mode of living of the host may be so adjusted that it cannot exist without its "symbionts." This master-servant relationship has doubtless reached its highest development in the insects. Of interest in this discussion, however, are the conditions in which the adaptations of the parasite to the host are as yet incomplete and the equilibrium between host and invader is quite labile.

The fields of helminthology, protozoology and botany offer many examples of harmless and highly adapted parasitism. Unfortunately, these balanced infections have aroused little general interest in the workshops of medical bacteriologists. The microbiologist, who intentionally administers huge doses of microörganisms in order to decide the pathogenicity of a disease agent for certain animals, has doubtless drawn and continues to draw erroneous conclusions. Fortunately, in recent years it has been appreciated that the massive infections, only too often supplemented by procedures which paralyze and deaden the resistance, have in no way imitated the natural conditions. Exposure experiments or the herd studies of Webster, Topley, Greenwood and Wilson more readily harmonize with the concepts of the parasitologist. This type of experimentation will answer more definitely the pertinent question as to which vertebrate or plant is the true host for a microbe or virus. To repeat, it is the animal or plant which assures the parasite a satisfactory permanent existence and since the physiology of the host harmonizes with that of the invader, the transmission to a new host of the same species is simple and permanently assured. Viewed biologically, an infection would pass phylogenetically through the following phases: from a free existence, the microbe, through an unequal symbiosis—parasitism *sensutrictu*—would reach the balanced and adjusted symbiosis.

The term symbiosis as originally used by the botanist, Anton de Bary (1879), denotes a condition of conjoint life existing between different organisms which are benefited in a varying degree by the partnership. As Nuttall has pointed out, the term symbiont, strictly speaking, applies equally to the partners. It has, however, come to be used also in a restricted sense as meaning the

microscopic, perhaps invisible member or members of the partnership in contradistinction to physically larger partners which in conformity with parasitological usage are conveniently termed the "hosts." On the other hand, symbiosis must be regarded as a condition of life balancing between two extremes—complete immunity and deadly infective disease. To be sure, it has been shown by botanists (N. Bernard, Magrou and others), whose theories are similar, that the factors governing immunity from parasites or symbionts are essentially the same for each partner. The struggle between the two partners had led in the course of time to a form of mutual adaptation. The symbiotic relationship of orchids and fungi has been experimentally investigated from this point of view, and it has been shown by N. Bernard that tuberisation is a sequel or a manifestation of an advanced adaptation of the plants to a communal life with fungi. It is worth considering that such structures as the mycetocytes, bacteriocytes and mycetomes in the insects are survivals of previous profound pathologic changes. How far this knowledge has any bearing on the various symbiotic stages which one observes in man and animals has not as yet been investigated.

It seems advantageous to discuss various other aspects of the problem of symbiosis as far as they pertain to the subject under discussion. Of particular interest are the important studies by Oehler, Pringsheim, Goetsch and others on the separation and recreation of algae symbiosis in paramecia, hydra, rhizopoda, etc. Dependent on whether their habitat is in fresh or in sea water, the endoplasm of these organisms may become occupied by *Chlorella* or *Zooxanthella*. An analysis of the factors involved in this type of symbiosis has shown that a separation of the partners may be achieved and that each may be cultivated independently of the other. However, this separation is no easy task and the many painstaking experiments which have been carried on attest to the striking tenacity with which the algae may adhere to the host. Various gradations of intimacy may be noted in one and the same species. Far more important than the separation of the plant from the animal is the causation of the symbiosis. There is absolutely no question that the immunity of the Algae against

the enzymes of the digestive vacuoles is of prime importance. As Pringsheim has demonstrated, it may be an inherent property of the Algae or it may be acquired within a few weeks. Certain Algae, despite systematic exposure to the invertebrate, remain susceptible. Equally important in the restoration of the symbiosis is the regulatory mechanism of the animal partner which controls the growth rate of the invaded Algae. Without this factor, the immune plant would readily overwhelm the animal as a parasite. Aside from this mechanism, one encounters as another host factor the disposition to accept the Algae as "symbionts." Certain species of *Hydra* freed from Algae, receive the plants and become green but within 10–14 days expel them just like ordinary food particles. In other cases the fresh water polyps are injured but following a massive and stormy multiplication and expulsion of clouds of Algae they ultimately recover. This form of elementary parasitism shows a remarkable analogy with the invasion of man and animals by unicellular plants. Further experiments indicate that the animal partner is, in a manner as yet unknown, profoundly impressed by a preceding symbiotic relationship. This property manifests itself in a greater receptivity; thus the customary partnership is rapidly renewed and balanced. Finally, the operation of the host-symbiont factors are rendered complex by the formation of physiologic races or types, morphologically indistinguishable but non-acceptable to the actinizoon or protozoon.

In connection with the study of a bacterial intracellular symbiosis in mollusks, the idea was expressed that this condition had its origin in true parasitism, probably disease. The same thought probably applies to the many and exceedingly complex symbioses observed among insects, irrespective of the doubts which have been voiced by Buchner, Reichenow, Florence and others. The comparative pathologist cannot recognize anything in the mycetocytes and mycetomes other than the survival of profound lesions induced by highly parasitic organisms. Such reasoning casts doubt on the widely expressed idea that the intracellular microörganisms are in a mutualistic relationship with their hosts. Until some of these forms have been cultivated

—an extensive new field for the bacteriologist acquainted with tissue culture technique—little can be said concerning their taxonomic position and their biologic significance. In fact, it is exceedingly doubtful whether a true state of symbiosis has been established. However, in order to avoid further confusion, it is advisable to retain the term "symbiosis" for the time being. Glaser, who critically compared the symbionts of the insects with the rickettsiae, correctly concludes that each of these intracellular elements originated among the invertebrates in the form of a disease, and that some of them are still in process of adaptation to higher animals in which they may produce definite diseases. The many examples of "inapparente infections" amply support his contention. Furthermore, he proposes a novel concept to explain the parasitic tendencies of *Rickettsia melophagi*. A gradual loss of characters adaptive to the invertebrate may be accompanied by a progressive modification leading to parasitism in the higher animals, particularly the mammals. His views are in harmony with present-day biological concepts and have numerous analogies as, for example, the evolution of the life cycle of the malarial plasmodium from a parasitic "symbiotic" state in the *Anopheles* to an advanced form of parasitism in man or birds. Finally, the biologically minded must agree with him when he casts doubt on the common belief that all new diseases may arise through sudden permanent modifications or variations of a disease agent instead of through a slow, orderly, evolutionary adaptation to the new hosts. It is not the purpose of this analysis to express an opinion relative to the importance of the "symbionts" as sources of growth-accessory factors, hormones, etc. That the "symbiosis" has a diversified function throughout the animal kingdom is fully recognized in all the speculative interpretations which have been published. However, Carter recently suggested that the phytotoxic secretions of certain coccids transmitting the spotting of pineapple leaves may be conditioned by the activities of the mycetome and its included symbionts. What bearing this hypothesis may have on the solution of certain insect borne diseases must remain a matter of conjecture.

Many of the facts briefly considered in the foregoing para-

graphs have prompted a number of bacteriologists to propound well sounding hypotheses and far reaching analogy deductions. For example, the commensalism of protozoa, ciliates in particular, in the rumen or stomach and in the coecum of herbivora has been designated as an endosymbiosis, which is active in the digestion of cellulose. This and the "symbiotes" themselves are thought to serve as nitrogen sources (for the horse it is estimated to be a quarter to one-third of the daily protein requirements) for the host. The implantation of bacteria into the buccal cavity and the intestinal tubes is compared with similar conditions in the insects and is known as episymbiosis. It has even been suggested that the early seeding of the mucosa of the mouth may be comparable with the bacterial contamination of the eggs of *Lagria hirta* while passing the smear glands of the urogenital tract of the insect. The new-born, passing through the vaginal canal, thus becomes the recipient of the maternal "symbionts." Additional examples of visionary comparisons could be cited but the few chosen emphasize again the unfortunate tendency to generalizations. From what has been outlined concerning symbiosis, it should be obvious that not one of the examples listed by Kolle and Prigge conforms to the fundamental definitions of a symbiosis. One could hardly support the idea that any one of the bacteria—pneumococcus, diphtheria bacillus, meningococcus, etc.—is in a mutualistic relation with the human host or that the gray rat is benefited by a partnership with *Trypanosoma Lewisi*. Since a true state of symbiosis has not been established, it is only proper to describe the organisms present on the mucous membranes or in the blood and tissues without evident disease as *commensals* and not as symbionts.

It is quite evident that such terms as "inapparente," silent infections or latent microbismus and commensalism describe a variety of processes. Since not infrequently the terminology has been applied without the necessary degree of care, an unfortunate state of confusion and contradictions has arisen. It is to the lasting credit of R. Doerr (1931 and 1934) to have clarified the problem, not only by proposing a tentative system in which the various independent processes or phases of an infectious disease

previously discussed may be arranged, but by listing some of the factors responsible for infections without disease. He clearly states that the principal criterion is the absence of *manifestations* of the disease which may be recognized either by direct observation or may find expression in man by a feeling of malaise. The opposite to manifest is latent; consequently the nomenclature will be greatly simplified if the various terms are classed under the collective designation "latent infection." Since "latency" is merely a clinical concept, it is quite permissible to use this term even when anatomical lesions inaccessible to direct observation or immunological and allergic reactions are recorded. Under certain circumstances one may even go further and describe a human being as "latent-infected" when general symptoms are absent and local reactions so exceptionally mild that the condition is recognized only by bacteriological procedures (for example a mild pharyngitis with diphtheria bacilli). Equally significant and applicable to a better understanding are the concepts with respect to the phases of an infectious disease. The state of a typhoid carrier is a *latent* one and not a manifest chronic infection irrespective of the fact that the presence of the typhoid bacillus may reveal itself clinically and anatomically (cholecystitis, focal nephritis). In clinical circles typhoid fever is defined as an acute, three-phased process which terminates with the restoration of the temperature to normal. With the onset of recovery, the clinical syndrome "typhoid fever" is passed and the ensuing carrier stage represents an entirely different condition which remains mostly latent and may only be recognized through a bacteriological examination.

In his latest discussion of the subject Nicolle (1935) now defines the "inapparente infection" as an infectious disease without symptoms. Its existence may be placed in evidence by direct or indirect laboratory methods, inoculation tests or serologic, immunologic procedures. He says, "L'infection inapparente perdra une part de son domaine au bénéfice de l'infection à symptômes. Réduites, elle demeurera la forme mineure des infections, celles qui échappe à la pratique usuelle du clinicien. L'inapparence n'a de sens que par rapport à la clinique."

From what has been said, it is obvious that "latent infections" may be either independent processes or phases of an infectious disease. This is clearly set forth in a descriptive system proposed by Doerr (1934) which makes no claim for completeness but merely outlines the main characteristics, their occurrence and their relationship to the clinically manifest processes.

SYSTEM OF LATENT INFECTIONS

(*1*) *Infections latent during their entire existence*

(a) *Cyclical processes.* This group covers the "infections inapparentes" of Nicolle.

(b) *Acyclical or chronic processes.* It is advisable to subdivide the group into (aa) the "silent microbismus" (tetanus, anaerobes, etc.) and (bb) the "quiescent infections" (tuberculosis, leprosy, etc.) irrespective of the fact that a clear distinction between the two forms is sometimes impossible.

(*2*) *Latency as a phase of an infectious disease*

(a) *As a precursor of the malady.* In the majority of infectious diseases, this period of latency may have a remarkably regular and consequently normal value while in others it may be either unknown or it may be exceptionally long—certain chronic infections, tuberculosis, actinomycosis, undulant fever, malaria (primary latency), rabies, etc. Diseases with pronounced tendency to latency rarely reveal regular incubation times. Undulant fever offers a great many striking examples in support of this statement: A young man has a febrile attack of one day duration; his blood yields a culture of *Brucella melitensis* but a frank clinical attack of undulant fever develops but 3 months later. In typhoid, cerebrospinal fever and diphtheria these so called *incubationary* (Nicholls) or *precocious* carriers may play an important rôle in the epidemiology of these diseases.

(b) *As a sequel of an infectious disease.* The state of latency in these so called "shedders" may be temporary and be terminated by spontaneous recovery or they may last for years or even during the entire life of the host. The word "shedder" has been employed since the proliferating microbes are, as a rule, eliminated

in the excreta and secreta. However, a carrier of "malarial gametocytes" is not a "shedder." Furthermore, distinctions have been attempted by designating as a "germ carrier" an animal or a human being who contracted the latent stage as an independent process without a previous visible disease. This confusing nomenclature is properly replaced by the significant designations "healthy" and "convalescent" carriers. Certain observers (Sacquepée and Doerr) believe that this differentiation is particularly valuable in diphtheria since they found the bacilli to persist longer in the convalescent than in those who had no recognizable clinical signs of the disease.

(c) *As an intermediary stage.* In the course of certain diseases with an intermittent or undulatory clinical course, periods of latency alternate with those of obvious disease. Dependent on the length of time which elapses between the manifest attacks, it is customary to speak of exacerbations or relapses, provided a reinfection may with certainty be excluded. In malaria, relapsing fever and certain septic processes, occasionally in *Brucella* infections, the sequence and duration of the latent and manifest phases follow with striking precision and are well regulated according to time. Malarial fevers offer an opportunity to analyze some of the factors which guide the latency. The febrile attacks coincide with the schizogony of the merozoites or agamonts and develop only provided the asexual forms reach a certain threshold which is in part dependent on the type of the malarial parasite (according to Ross, at least 140 quartan, 200 to 300 tertian and 600 to 3,000 tropical parasites per 1 cc. must be present before a febrile attack is induced). In vaccination malaria, not less than 14,000 tertian parasites per cubic centimeter cause fever. From the many speculative hypotheses, which have been advanced in order to explain the febrile paroxysms as an aftermath of the schizogony, the concept of poison formation by the interaction of agamonts and cellular detritus on the blood plasma (Doerr), and the phagocytosis of the parasites by the reticulo-endothelial cells (Taliaferro) deserve serious consideration. The investigations of Cannon and Taliaferro on the cellular basis of resistance to both avian and simian malaria suggestively

illuminate the rôle of the macrophage-lymphocyte system in the mechanism of latency. These superb studies have shown that in the course of these infections a certain percentage of the parasitized red blood cells are phagocytized by the "hemophages" of Kyes and rendered inert. Peculiarly, this function is not continuous but synchronizes with the development of each new generation of parasites. The periodicity of malarial fever may well be intimately connected with this mechanism. During latency against plasmodia, the host acquires some factor or factors which specifically cause the macrophages to ingest the parasites. Whether this altered reactivity of the reticulocytes is supported by a humoral factor in form of a tropin is not as yet settled. Similar in many ways is the mechanism in relapsing fever. Autopsies on children (Bykowa, 1926) or animals infected with *Spirochaeta recurrentis* show a marked hypertrophy of the reticulo-endothelial apparatus. An interplay between lytic antibodies and the mesenchyme cells guides the periodicity of the fever attacks. Protection of the spirochaetes within the cells and development of antibody-fast variants may lead to latency. In many septic diseases (undulant fever, various streptococcic infections, etc.), it has been noted that, as a rule, the causative microbes are less likely to be isolated from the blood during an afebrile than a febrile period. Then again, in certain animal diseases, the phenomenon of "microbial showers," by no means explained, merely indicates that in all probability the bacteria multiply irregularly. Some clinicians, by analogy with protozoan infections, attribute this behavior to growth cycles. However, the evidence, which gradually accumulates, involves the cellular activity of the host and not of the parasites.

The diverse experimental studies to convert a latent infection into a manifest process and *vice versa* offer many suggestions relative to the *causes of latency*. By splenectomy, latent plasmodial infections of apes (Gonder and Rodenwald) and piroplasmoses (dog, sheep and horses) have been changed into severe, even fatal infections. The entire *Bartonella* problem received a great impetus from the systematically planned removal of the spleen in rodents. The same principle applied to other latent protozoan infection has been even more fruitful. A *Trypanosoma Lewisi*

infection of the gray rat runs, as a rule, a benign and clinically latent course. Regendanz and Kikuth (1927) showed that in splenectomized rats the multiplication of the trypanosomes was frequently not retarded. In fact, the infection was thus transformed into a manifest process and frequently terminated fatally. Similarly, the extirpation of the adrenal by Marmorston, Gotterman and associates was followed by a like result in approximately 70 per cent of the experiments.

To the disappointment of many a worker, latent bacterial or virus infections in laboratory animals have been recognizable by the injection of diverse agents. Carriers of *Bacterium leptisepticum* may be made recognizable by placing silver nitrate or dyes into the nostrils of the suspected rabbits. The microbian associations (Microbio de salida or Microbes de sortie of Maurice Nicolle) with protozoa in virus diseases belong to this group. With increasing frequency, "latent infections" are converted into visible diseases by transferring organ material of various animals to new hosts. One recalls the discovery of the virus III of rabbits, the encephalitis virus of mice and the protozoon *Encephalitozoon cuniculi*. Saprophytic viruses are discovered in cultures (Barnard). *Vice versa*, as already detailed, manifest infections on a large scale and quite successfully have been transformed into latent stages by shifting the field of action from one host to another species. Although theoretically of greatest interest and subject to extensive investigation, these experimental metamorphoses contribute little to the understanding of the problem of latency in man.

Concerning the mechanism or the cause or causes of latency, the available knowledge is exceedingly fragmentary. For a few cases, a satisfactory explanation may be offered. The *localization of the infectious process* may condition the latency. A small tuberculous or fungoidal focus in a lymph-node frequently does not provoke clinical signs. The commensalistic existence of meningo- and pneumococci on the mucosa of the respiratory tract remains "symptomless" until the invasion of certain organs is followed by the typical clinical syndromes of cerebrospinal fever or lobar pneumonia.

Certain forms of latency are, in part at least, influenced by the

behavior of the disease-producing agent. The mechanism of the resting tetanus or gas gangrene infection has been explained as a phenomenon of growth inhibition which takes place at the moment the microbe is transmitted. In contrast to this primary lack of vegetation of the spores, a secondary retardation in the growth of bacteria in encapsulated abscesses, scarred or calcified tissues may be equally responsible for the absence of symptoms. A small number of infective agents, in part inhibited, may never multiply to a level which incites the clinical reactions. Under the circumstances, the infection may remain latent either through a long incubation time or for variable periods between primary attacks and relapses. In the considerations which are given to the carrier problems, one meets not infrequently the hypothesis that the latency was the result of a microbe of low or even non-pathogenicity. It is easy to assume fluctuations in virulence of the causative organism but its relationship to latency can *rarely* be proved. When the pathogenicity is associated with a specific toxin, successful correlations have been established. Thus, occasionally healthy diphtheria carriers may harbor organisms of relatively low toxicity. In fact, in rare instances, non-toxic strains have been isolated. However, as a whole there is no evidence that a true *Corynebacterium diphtheriae* loses completely its ability to produce toxin, and as such becomes an organism particularly endowed to create carriers. On the other hand, there are authenticated reports in the field of latent microbian and virus infections which conclusively prove a persistent and uniformly high pathogenicity during a very long sojourn of the disease agent in the tissues of the host. Under the influence of increasing but incomplete knowledge on the variability of bacteria, it is by no means surprising to note that latency is believed to be the result of a transformation from a pathogenic to a non-pathogenic variety. The frequent demonstration of rough variants in the excreta of typhoid-paratyphoid carriers lends support to these hypotheses. In fact, certain groups encourage the concept that these variants are stages of a continuous adaptive process leading ultimately to a harmless symbiont. Others, making use of the unsolved problems of life cycles, suspect

changed facultative invisible forms of the disease agents to be factors responsible for the latency in tuberculosis and syphilis. The effort is thus made to incriminate only the microbes. Despite the ubiquity of certain bacteria and the disease induced by them, not everybody becomes a carrier or acquires a latent infection. Without more precise information relative to such vague terms as "infectiousness" or pathogenicity, little progress will be made in the understanding of latency.

The mechanism known as "organotropism" by which is selected the tissues in which the parasites localize themselves and the many factors which control their growth and multiplication have been analyzed. Technical difficulties have prevented studies with plant parasites and viruses, but for certain macroparasites, *Trichina spiralis* and protozoa (malaria), the selective organ localization has been proven. Equally encouraging are the preliminary investigations on the influence of a trypanosome infection on the protein, carbohydrate and fat metabolism of certain rodents. It is assumed that the parasites disturb the metabolism and that, in turn, the abnormalities affect the nutrition and multiplication of the protozoa living in such a milieu. A study of *Pasteurella pestis* infections in hibernating rodents with profoundly changed metabolism discloses interesting and important facts which may help to explain certain causes of latency.

In the course of an infection, the environment to which the parasite is exposed changes continuously. It is certain that the disease agent is subjected to influences of varying intensities during the incubation time, the onset, the peak and the healing of the infection. Adaptations are suspected and newer studies attempt to demonstrate all kinds of transformations although for technical reasons, it is usually impossible to prove conclusively their existence in the living host. The solution of such intricate problems requires expert knowledge of modern experimental-pathological methods, and should not be attempted by those who merely know the "test-tube" and not the "animal" phases of a microbe.

Infectiousness and pathogenicity are relative conditions which are not dependent on the parasite alone, but in more important

degree, on the individual, racial and species characteristics of the host. Aside from a disposition for a manifest disease, the host in all probability possesses an equally important yet independent susceptibility for infections without pathological consequences. *Latency is conditioned by the behavior of the infected macroörganism.* For the sake of completeness, the various possibilities may be briefly noted. The possession of one factor or factors, which antagonize or neutralize the injurious effect of the microbe, for example, antitoxin content of the blood in a diphtheria carrier, may be important. Or a preceding infection of like etiology or phases of the disease may change the reactivity of the organism to such an extent that a reinfection or persistent infection may appear or remain in a clinically latent state. Since these modifications in the clinical course are the result of immunity reactions, Doerr has proposed the term "immunosatoric latency." Why the defensive mechanism remains incomplete and then suddenly ceases to function so that the disease becomes manifest as a relapse or is so intensified that a carrier state after months and years is promptly healed has not been explained. It must be frankly admitted that the method by which the host participates in these reactions is not known. These resistance variations should be correlated with phagocytic activity. Studies in this direction are obviously indicated. Latency is rather frequent in the infections in which the phagocytic mononuclear cells actively participate in the defense, or furnish the environment most conducive toward a favorable development of the infective agent. Extensive studies on the histopathology of *Brucella* infections and psittacosis suggest an experimental approach to a clearer understanding of the immunosatoric latency.

Dependent on certain conditions of the host are those forms of latency which already as primary infections remain free from clinical signs. The latent yellow fever and typhus fever of children, dengue in adults, typhoid bacilli in the blood of healthy persons and various *Rickettsia* infections in laboratory animals belong to this group. Since the effects of active or passive immunity can with certainty be excluded in the typhus infections of laboratory animals, it is strange that this state of individual

tendency to "latent infection" has not been correlated with the operation of genetic factors. Even the immunosatoric latency is in part influenced by these forces. Competent observers have in recent years pointed out that focalization and chronicity of a microbian disease process is not merely conditioned by individual immunizing subinfections but ultimately may be traced to the selection of resistant generations (Hirzfeld). Though the analysis of constitutional immunity is in its infancy, the data thus far available suggest that latency is often the expression of the host's inheritance rather than the variability of the disease agent. However, it must be stated with frankness that it will be impossible to list or discern more in detail the causes of latency until accurate information concerning the factors which promote or suppress symptoms of an infectious disease is available. The problem of "inapparente infections" is a part of the unsolved problem of pathogenicity. In its broader aspects it is the key to the understanding of latent epidemization and as an evolutionary phase participates in the rise and decline of certain diseases. The results of the scientific investigations of this important problem will doubtless furnish a rationale for the natural fluctuations of epidemics which now cannot be completely explained either through the frequency of the manifest infections or by the variability of the causative agent. Finally, this analysis has shown that a comprehension of mass disposition is intimately dependent on a clearer understanding of individual disposition and disease symptomatology.

THE GROWTH OF A BUTANOL CLOSTRIDIUM IN RELATION TO THE OXIDATION-REDUCTION POTENTIAL AND OXYGEN CONTENT OF THE MEDIUM

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INTRODUCTION

One of the fundamental advances in biology was the discovery of anaerobic life by Pasteur. Since then, anaerobiosis has been found to be quite general, and we have come to realize that oxidation, in its broadest sense, is more fundamental than mere combination with oxygen. As a result, we have now a clearer insight into some of the processes of anaerobic life.

Among the modern contributions to the study of the relations between the oxidation-reduction potential of the medium and the growth of anaerobic bacteria, we may mention those of Quastel and Stephenson (1926), Aubel, Aubertin and Génevois (1929), Fildes (1929), and Plotz and Geloso (1930). *Clostridium sprogenes* and *Clostridium tetani* were the principal organisms investigated, and the conclusions were that the range of potentials at which these organisms grow corresponds to rH 0 to 12 or 0 to 14.

There is a tendency at the present time to generalize the knowledge gained from the study of anaerobes to aerobic processes. This tendency probably has its origin in the brilliant investigations of Wieland (1912) who brought about the formation of acetic acid from alcohol by the acetic bacteria when oxygen was replaced, as a hydrogen acceptor, by quinone or methylene blue.

More recently Allyn and Baldwin (1930, 1932) reported profound influences of oxidation-reduction potentials of the culture medium on the initiation of growth and on the character of growth of several members of the genus *Rhizobium*. On the addition of

a small amount of potassium permanganate to the medium, they also reported a slight evidence of cell proliferation under anaerobic conditions.

On the other hand, Knaysi and Dutky (1934) were unable to secure growth of *Bacillus megatherium* when oxygen was replaced by ferric ammonium citrate, although the potential of the medium remained within a range in which the organism produced luxuriant growth in the presence of oxygen.

In the present investigation we have attempted to study the relation of the potential of the medium and of its oxygen content to the growth of a spore-forming anaerobe belonging to the group producing butyl alcohol. This clostridium is sufficiently different from the two anaerobes previously mentioned to make such a study desirable. It was isolated from soil, but is not a starch-fermenting organism and, therefore, is not identical with *Clostridium acetobutylicum* (Weizmann).

EXPERIMENTAL

1. *Medium used.* The basic medium used throughout this work was a broth containing 1 per cent peptone, 0.3 per cent beef extract, and 0.5 per cent d-glucose. The medium was distributed in 20 cc. portions in large test tubes, 25 mm. in external diameter, the height of the liquid in the tube being nearly 50 mm. After sterilization, the pH was about 6.8, and did not change until considerable growth took place. This basic medium was used as such or after having been made 0.005, 0.0025 or 0.0001 molar with respect to potassium ferricyanide, a compound employed by Cohen (1931) and possessing excellent properties for this kind of work.

2. *Potentials of the sterile medium.* The aerobic oxidation-reduction potentials of the sterile basic medium and of the same medium made 0.005 molar with respect to potassium ferricyanide are shown in figure 1. Figure 2 shows the potentials of these same media when nitrogen gas was continuously bubbled through them, and curve *A* of figure 3 represents the potentials of the basic medium in partial vacuum.

All potential readings were made at 37° to 38°C. and are re-

ferred to the normal hydrogen electrode. Anaerobic conditions were produced by continuous bubbling of nitrogen gas filtered through sterile cotton, although we realize, as did Plotz and Geloso (1930), its inefficiency in creating anaerobic environments. However, we were especially interested in bubbling a gas like nitrogen in order to keep the medium stirred and remove the possibility of localization. The small amount of oxygen which

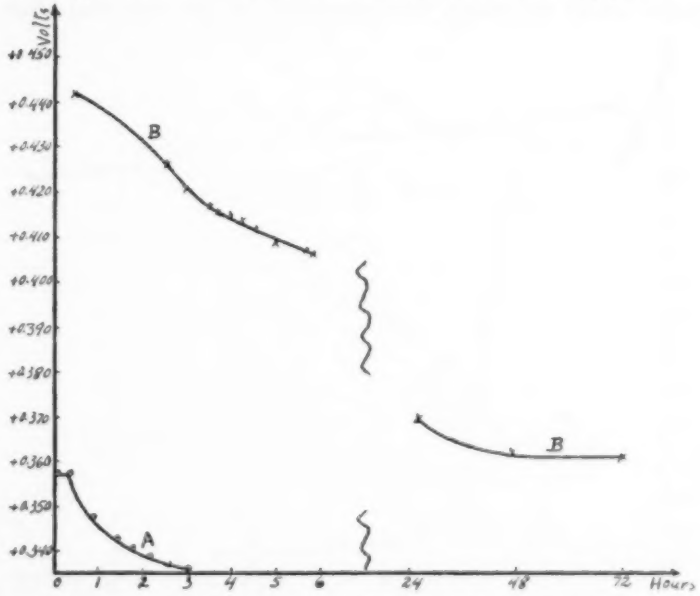

FIG. 1. TIME-E_h DIAGRAMS OF THE BASIC MEDIUM (*A*) AND OF THE 0.005 MOLAR POTASSIUM FERRICYANIDE BROTH (*B*) UNDER AEROBIC CONDITIONS

might have been present did not interfere with the growth of the organism used which, unlike the two anaerobes mentioned above, tolerates a fair amount of oxygen as will be shown later. The partial vacuum was produced inside of the apparatus previously described by us (1934).

Numerous experiments were performed, practically all showing excellent agreement with the few typical ones illustrated in the graphs.

3. *Remarks on potassium ferricyanide.* When potassium ferricyanide is added to the sterile broth it is reduced at a regular rate. The potential of the medium gradually drops from a relatively high positive value to a value determined by the concentration of ferricyanide, the nature of the medium and the conditions under which the mixture is placed. At a given temperature and for a given initial concentration of the ferricyanide, it is possible to predict fairly accurately the magnitude of the potential after a

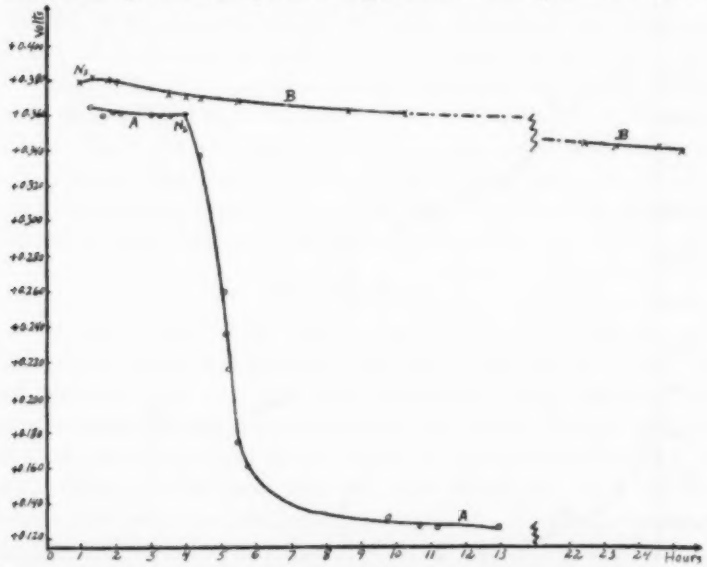

FIG. 2. TIME-E_h DIAGRAMS OF THE BASIC MEDIUM (*A*) AND OF THE 0.005 MOLAR POTASSIUM FERRICYANIDE BROTH (*B*) WHILE NITROGEN GAS WAS BUBBLED
N_2 indicates the point at which nitrogen was bubbled

given time. This was taken advantage of in studying the effect of potential on the growth of the organism, as will be seen later.

The ferricyanide medium gives very definite potential readings, and a noble metal electrode immersed in that medium is not subject to polarization under ordinary conditions. In addition, potassium ferricyanide has a high poising effect on the medium as is shown by a comparison between its potentials under aerobic and anaerobic conditions with and without ferricyanide. How-

ever, if the salt is completely reduced, either because of too low a concentration or on account of to much growth, the above two advantages may be removed.

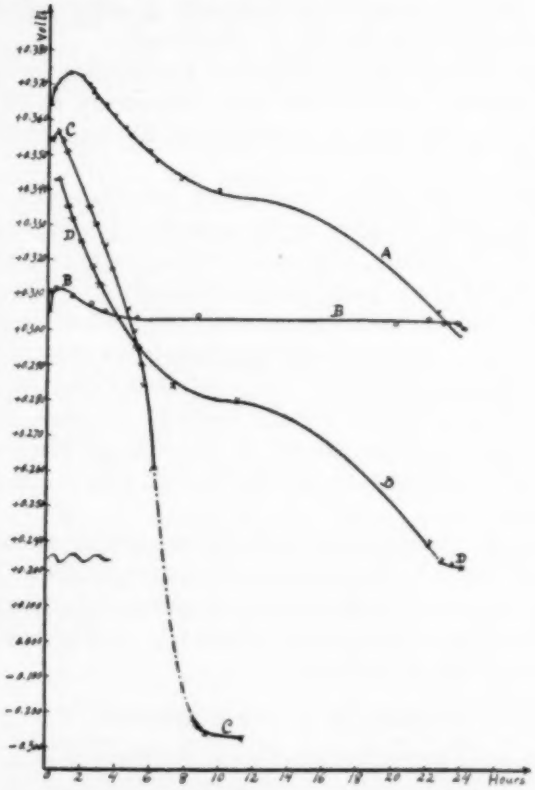

FIG. 3. TIME-E_h DIAGRAMS IN PARTIAL VACUUM

A, for the sterile basic medium under an air pressure of 297 mm. of mercury. *B*, for the basic medium inoculated with 0.1 cc. of a 24-hour-old culture under an air pressure of 285 mm. of mercury. *C*, for 0.0001 molar potassium ferricyanide broth inoculated with 0.1 cc. of a 24-hour old culture, under an air pressure of 296 mm. of mercury. Excellent growth is shown by the very negative potential attained. *D*, for the sterile 0.0001 molar potassium ferricyanide broth under an air pressure of 289 mm. All at 38°C.

4. *Growth in the basic medium.* The organism does not grow in our basic medium under aerobic conditions, but gives profuse

growth if the medium is heated and cooled previous to inoculation. The volume of the inoculum, unless otherwise stated, was 0.1 cc. of a 24-hour old culture in another tube of the basic medium. The potential of a vigorously growing culture may drop to the neighborhood of $E_h = -0.265$ volt.

Under partial vacuum, the organism was constantly inhibited by an air pressure of 270 to 297 mm. of mercury at 38°C. (see curve *B*, fig. 3), but grew at a pressure of 200 mm. at the same temperature.

5. *Growth in the medium containing potassium ferricyanide.* The organism does not grow in the potassium ferricyanide broth under aerobic conditions, but grows well under anaerobic conditions. If the tubes are inoculated immediately after the addition of the ferricyanide, growth appears sooner in the lower concentrations of the salt. In one of our experiments we observed strong turbidity and gas production at 37°C. in 0.0001 molar ferricyanide broth in 24 hours, in 0.0025 molar broth in 36 hours, and after 52 hours when the concentration of the ferricyanide was 0.005 molar, all tubes having been inoculated with 0.05 cc. each of the same culture.

One of the interesting things observed was growth of the organism in 0.0001 molar broth under an air pressure of 289 to 296 mm. of mercury, when no growth was ever obtained at similar pressures in the basic medium (fig. 3, curves *B* and *C*). A discussion of this observation will be given later.

FURTHER DATA AND DISCUSSION

Space does not permit publication of the mass of data bearing on the various points under consideration. However, the curves of figures 1 to 4, supplemented by additional data, will, we hope, demonstrate the logic of our conclusions.

Figure 1, *A* shows that our basic medium has an aerobic potential slightly more positive than +0.335 volt at 37°C. Under these conditions the butyl alcohol organism is unable to grow in that medium. Under anaerobic conditions, the potential of the medium drops to about +0.125 volt (fig. 2, *A*), and the organism grows well. However, this does not permit us to say whether it

was the removal of oxygen or the drop in potential that made conditions suitable for growth.

If now we can keep the potential high enough in the absence of oxygen, we shall have separated the rôle of oxygen in the oxidation-reduction system from its specific rôle as oxygen. This can be done by the addition of a certain amount of potassium ferricyanide.

Let us consider for the present the case of 0.005 molar potassium ferricyanide. When first added, the ferricyanide gives the medium a potential of over +0.440 volt; but the potential drops gradually and remains for sometime in the neighborhood of +0.360 volt under aerobic conditions (fig. 1, *B*). This is not very far from the potential of the basic medium under the same conditions.

Under anaerobic conditions, the potential drops still further, but very slowly (fig. 2, *B*). After a long enough time, the ferricyanide may be completely reduced. If left in the vacuum for 2 days at 37°C., however, the potential will still be found close to +0.320 volt. This shows the excellent poising action of 0.005 molar potassium ferricyanide. It has been already mentioned that growth does take place in such a medium under anaerobic conditions, but requires a rather long time. So far, the data point to oxygen as the sole inhibiting factor, for the retarded growth in the presence of the ferricyanide can be explained by assuming some toxic effect of the salt other than potential. However, the following observation will modify our views.

We had repeatedly observed that growth never occurred in 0.005 molar broth until the potential of the medium dropped slightly below +0.335 volt. Further, we were able to hasten growth considerably by incubating the sterile medium in the vacuum until its potential dropped before inoculation, and the degree to which growth was hastened varied inversely with the potential. The following experiment shows this most impressively.

One sterile broth tube with 0.005 molar potassium ferricyanide was incubated under the vacuum. At the end of 28 hours, a tube of the basic medium was made 0.0025 molar with respect to

potassium ferricyanide and placed in vacuum for a few minutes. Before the desiccators were opened, they were filled with nitrogen at atmospheric pressure. Both tubes were warmed to 37°C. and inoculated, each with 0.1 cc. of a 24-hour old culture at 37°C. in the basic medium. Immediately afterwards, the tubes were

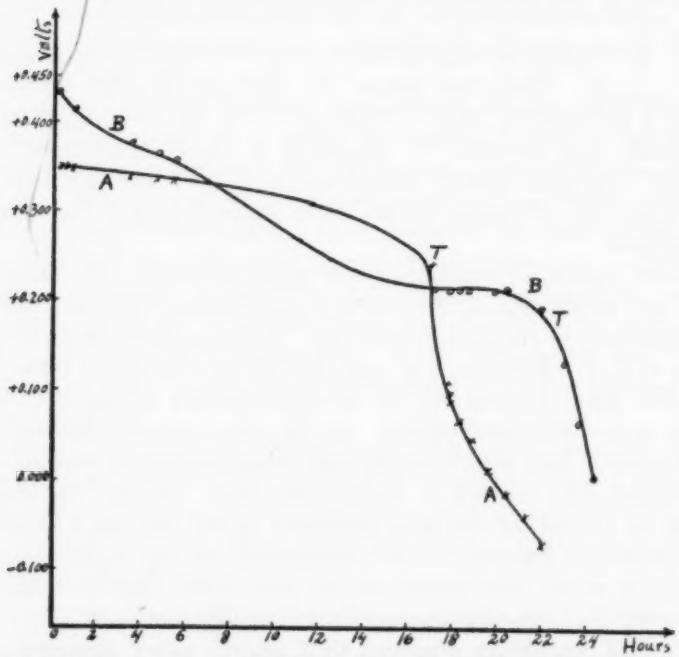

FIG. 4. TIME-E_h DIAGRAMS OF 0.005 (*A*) AND 0.0025 (*B*) MOLAR POTASSIUM FERRICYANIDE BROTH INOCULATED WITH 0.1 CC. OF A 24-HOUR-OLD CULTURE
The 0.005 molar broth was incubated at 38°C. in the vacuum before inoculation. The 0.0025 molar one was placed in the vacuum only a few minutes before inoculation. Nitrogen gas was bubbled immediately after inoculation. *T* indicates the point at which definite turbidity was seen.

connected with the potentiometer and nitrogen was bubbled through them. The initial potentials were +0.3472 volt for the 0.005 molar tube and +0.4330 volt for the 0.0025 molar one. Turbidity was observed in the first tube after 17 hours, while, in the second, turbidity was first seen after nearly 23 hours. The

potentials corresponding to the appearance of turbidity were +0.241 volt for the first tube and +0.195 volt for the second. This difference is due to a difference in the poising action of the ferricyanide because of the difference in concentration. The significant thing is, however, that the difference between 17 and 23 hours is very nearly the time it took the 0.0025 molar tube to reach a potential of +0.3472 volt. This experiment is illustrated in Figure 4.

The above observation is not an absolute proof of the importance of potential, but furnishes strong evidence in its favor.

Let us now consider the experiments performed under reduced air pressure.

1. It has already been said that the *Clostridium* under investigation does not grow in our basic medium under an air pressure in the neighborhood of 290 mm. of mercury at 38°C. Now the potential of the basic medium under such conditions is about +0.300 volt. It must, therefore, be concluded that the positive potential produced by oxygen is much more effective than that produced by potassium ferricyanide in inhibiting the growth of the organism. This point will be discussed later.

2. A very interesting point mentioned above is the ability of the *Clostridium* under investigation to grow under an air pressure of about 290 mm. of mercury at 38°C. when the medium is made 0.0001 molar with respect to potassium ferricyanide (fig. 3, *C*). It was our practice to incubate the tubes for a certain length of time after the addition of the ferricyanide in order to insure sufficient drop in the potential.

In one experiment, the ferricyanide had been added two days previously. At the start of the experiment and soon after a partial vacuum was established, the initial potential of the sterile medium was +0.3422 volt. Five hours later, it was +0.294 volt, and after 24 hours it had attained +0.215 volt (fig. 3, *D*).

In order to find out whether the lower potential was alone responsible for the growth, or whether this lower potential had caused a reduction in the oxygen content of the medium, it was thought that oxygen determinations at atmospheric pressure

should bring out any possibility of difference in oxygen content. For this purpose several tubes of the basic medium and several others of the same medium containing potassium ferricyanide in 0.0001 molar concentration were incubated at 37°C. for one day, then at 30°C. for six days. The average of two oxygen determinations gave 0.045 and 0.019 cc. per 20 cc., respectively, of the basic medium and of the medium containing potassium ferricyanide. Again these results suggest the possible danger of drawing conclusions from mere potential measurements in a problem of this sort.

SOME THEORETICAL CONSIDERATIONS

To summarize our conclusions from a previous paper on the aerobic *Bacillus megatherium* (1934) and from the present investigation of the anaerobic butyl alcohol organism, we may state: In the media and under the conditions used, oxygen seems to be the limiting factor in the growth of *Bacillus megatherium*. In the absence of oxygen, no growth takes place, although the potential of the medium is kept within a range quite suitable for growth when oxygen is available. Suitable potentials under anaerobic conditions were obtained by the addition of ferric ammonium citrate to the medium. In other words, ferric ammonium citrate could not replace oxygen for *Bacillus megatherium* under the conditions used.

The present investigations show that the butyl alcohol clostridium is inhibited when oxygen is present at a partial pressure that causes in the medium a potential of about +0.300 volt with respect to the normal hydrogen electrode, but that it takes a potential in the neighborhood of +0.335 volt to cause inhibition in the absence of oxygen. The substance used to keep the potential relatively high under anaerobic conditions is, in this case, potassium ferricyanide. We secured strong evidences that the potential, as such, is quite important with this anaerobic organism, although oxygen seems to be particularly efficient in stopping growth.

Let us now examine these factors in the light of what is known about their relation to the nutrition of living cells.

Significance of the potential. In the words of Cannan, Cohen and Clark (1926), the fundamental importance in the potential "lies in its possible relation to the intensity levels of the work function which determine the direction of oxidative and reductive reactions." This can be illustrated by the brilliant analysis made by Wurmser (1923, 1930) of the reactions resulting in the synthesis of alanine from pyruvic acid and ammonia in the presence of glucose. On purely thermodynamic grounds, Wurmser concluded that such a synthesis is possible only in a milieu with a potential corresponding to rH $\leqslant$ 20. At a more positive potential, glucose would be oxidized by molecular oxygen and not by pyruvic acid. The result would be respiration instead of synthesis. The figure he obtained agrees quite well with the direct determinations of potential of the protoplasm by micro-injection.

In other words, a certain potential is a *necessary* condition for the realization of a given reaction.

The question is now: is the potential a *sufficient* condition for the realization of the various reactions involved in the growth of living cells? In the light of what follows, the answer to this question will be in the negative.

Disregarding our results with *Bacillus megatherium*, we have in the work of Wieland himself, a definite basis for that answer. Wieland (1932) says: "The activated hydrogen of a substrate, produced by a dehydrogenating enzyme, cannot be transferred to any acceptor which is suitable on grounds of affinity alone. There is a very marked selectivity as regards acceptors.... Thus, the acetic acid bacteria cannot bring about the dehydrogenation of either alcohol or acetaldehyde, when peroxides or disulphides are substituted for the previously discussed acceptors (i.e., quinone and methylene blue)." Persulphates, diethyl peroxides, cystine, dithiodiglycollic acid and hydrogen peroxide were also unsuitable. Another case to the point is the failure of Wieland to maintain the activity of frog heart in Ringer's solution containing methylene blue in the absence of oxygen (see Wurmser, 1930). The reader is also referred to a paper by Quastel and Wooldridge (1929) where Quastel modified his earlier view. We might add that these experimental results are predictable

from thermodynamics which can specify the conditions under which a reaction can take place, but do not predict that it will take place.

The rôle of free oxygen. Free oxygen may play one or both of two rôles. First, it may act through its ability to establish a certain level of potential, beneficial or harmful for the carrying out of life processes. Second, it may furnish energy for independent oxidations or for coupled reactions, sometimes entering into the composition of the products of reactions in which it participates. In its first rôle, it is probable that it could be replaced by certain other substances. In its second rôle, it may or may not be replaceable by other substances.

Relation of the potential of the medium to that of the cell. At the present time we know little about the relation of the potential of the medium to that (or probably those) of the cell. Wherever life reactions take place, we have evidences that the potentials registered in a bacterial culture are due to these reactions, and therefore, should give us a clue to the oxidation-reduction status of the cell. Whatever the case may be, it seems to be demonstrated that the potential of the medium has, within certain limits, a profound influence on the type of growth, and, outside these limits, on the growth ability of the cells.

Remarks on the high efficiency of oxygen in inhibiting the growth of anaerobes. McLeod and Gordon (1923) attribute the toxic effect of oxygen on anaerobes to the accumulation of hydrogen peroxide in the absence of catalase. On the other hand, Sherman (1926) calls attention to the fact that certain propionic bacteria which may be classed as anaerobes are especially vigorous producers of catalase. Quastel and Stephenson (1926) have shown that the addition of catalase to the medium does not remove the toxic effect of oxygen, while reducing substances do so. It seems likely that the formation of hydrogen peroxide is due to the presence of oxygen as such, but that the accumulation of that product is made possible by the high potential induced by the oxygen. Thus, we may account for the existence of catalase-producing anaerobes, as well as the greater tolerance to oxygen possessed by such organisms as the propionic acid bacteria. In

addition to this, an important factor may be the possible difference in accessibility of the regions of the cell where various chemical processes take place to substances like potassium ferricyanide and oxygen.

SUMMARY

The growth of a butyl-alcohol-producing clostridium has been investigated in relation to the oxygen content and the oxidation-reduction potential of the medium.

In the absence of oxygen, the organism grows in a medium with a potential slightly below +0.335 volt, compared to the normal hydrogen electrode. Such a potential was maintained under anaerobic conditions by the addition of potassium ferricyanide.

On the other hand, an oxygen pressure corresponding to a potential of about +0.300 volt inhibits growth of the organism.

An attempt to explain this greater efficiency of oxygen is made, and a theoretical discussion of the rôle of oxygen and of the importance of potential is given.

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THE EFFECT OF ATMOSPHERES OF HYDROGEN, CARBON-DIOXIDE, AND OXYGEN, RESPECTIVELY, AND OF MIXTURES OF THESE GASES ON THE GROWTH OF BACILLUS SUBTILIS

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The literature on the rôle of various gases in bacterial respiration is quite conflicting. That free oxygen is consumed and carbon-dioxide given off by aerobic organisms has long been known. Within recent years carbon-dioxide has been credited with possibly being a catalyst or even a source of carbon for *Brucella abortus* (Smith, 1924). That this gas appears to be beneficial in primary isolation of certain organisms has also been shown (Chapin, 1918; Cohen and Fleming, 1918; Huddleson, 1921). On the other hand investigators have contended that carbon-dioxide merely serves indirectly to decrease the oxygen tension or increase the acidity of the medium (Gates, 1919; Kohman, 1919; St. John, 1919). Only recently have studies been made on the gaseous requirements of aerobic spore-bearing bacilli. Rockwell (1921, 1923), Valley and Rettger (1926), and others have reported that carbon-dioxide plays a more important rôle in respiration and initiation of growth of aerobic spore-bearers than had previously been thought. The experiments reported here were carried out in an attempt to determine the effect of oxygen, hydrogen and carbon-dioxide on the spore germination and vegetative cell multiplication of *Bacillus subtilis*.

PREPARATION OF THE GASES

Hydrogen. A 2-liter flask was tightly stoppered with a 2-hole rubber stopper provided with glass and rubber tubing con-

nections. By applying gentle suction to one arm, a 10 per cent solution of NaOH was aspirated into the flask until the entire vessel and both glass arms were full. After the connections had been clamped tightly, the flask was inverted and connected to a commercial hydrogen tank. All connections were filled with water to displace the air. Both clamps were removed and the gas was allowed to enter the flask, displacing all but approximately 200 cc. of the alkali solution. Once more the outlets were clamped and the flask placed in an upright position. Suction was again applied and 120 cc. of a freshly prepared strong aqueous solution of pyrogallic acid was aspirated into the flask without introducing any air. By shaking the flask vigorously for 15 minutes it was possible to produce a fine spray of the washing solution, thereby insuring the greatest washing surface for the gas. At the same time the amount of O_2 absorbed could be estimated from the color of the pyrogallol. The gas was never used when the washing solution had turned dark. Had any traces of CO_2 been present it should have been absorbed by the alkali. It is believed, therefore, that the H_2 gas, so purified, was free from both O_2 and CO_2.

Carbon-dioxide. A flask similar to that described for the H_2 purification was prepared. Into this was placed 75 grams of white phosphorus. The flask was quickly filled with water and stoppered. Carbon-dioxide was introduced from a commercial cylinder displacing all but 200 cc. of the water. By placing the flask on its side, the water uncovered the phosphorus, which, upon exposure to the free O_2 present as an impurity, promptly smoldered. White clouds of P_2O_5 and P_2O_6 formed which combined slowly with the water to form phosphoric acid. Placing the flask in hot water hastened the combustion process. Usually $2\frac{1}{2}$ to 3 hours had to elapse before complete burning of the O_2 had been effected and the combustion products had combined with the water. When ready for use the CO_2 was clear and odorless.

Oxygen. In the preparation of O_2 special efforts were made to eliminate CO_2. The delivery tube of an oxygen generator was connected to the reservoir which had been previously filled

with 10 per cent NaOH solution. The alkali was displaced until only 200 cc. remained. After the arms of the flask had been clamped, it was thoroughly shaken for 15 minutes.

When it was desired to withdraw any of the gases into the culture flasks, one of the reservoir outlets was placed under water in an open graduated vessel. The clamp on the other outlet was left in place until the connections with the culture flask had been made and the line exhausted. The volume of gas drawn into the culture vessel could then be measured accurately by observing the amount of water drawn out of the graduated vessel into the reservoir.

TECHNIQUE

Eight-ounce Blake bottles were used as culture flasks. These were strong enough to admit the use of a high vacuum and had sufficient volume so that gaseous changes due to the action of the bacteria themselves were inappreciable. These flasks also permitted easy observation of the bacterial growth.

The medium used in most of the experiments was an ordinary meat-extract agar. A buffered medium having the same composition as above with the addition of 1 per cent K_2HPO_4 was used in many of the experiments with CO_2. The reaction of the media, determined colorimetrically, ranged from pH 5.9 to 7.8 in the different experiments.

Spore-bearing inocula were prepared by shaking a bit of growth from a 4-day slant culture in sterile physiological saline until an even suspension was formed. A fresh suspension was prepared for every experiment. An attempt was made to keep the successive suspensions of uniform density so far as could be judged by the eye.

For vegetative cell inocula a 2-hour bouillon culture was used. This was prepared by making successive transfers in bouillon, incubating each subculture for 2 hours. The absence of spores was verified by the failure of the suspensions to withstand heating at 80°C. for 30 minutes.

Approximately 20 cc. of melted agar were poured into each bottle. The agar after cooling to 45°C. was seeded with two

loopfuls of inoculum. A 2-hole rubber stopper with inlet and outlet tubes was then pushed into the bottle over the cotton plug. The inoculated bottle was then placed in a water bath at 45°C. to prevent the agar from solidifying. One outlet was connected to an oil-sealed rotary pump capable of reaching a vacuum of less than 0.1 mm. of mercury. All joints and connections were heavily coated with a thick layer of shellac to prevent leakage.

The vacuum pump was now started and the culture bottle and the tube connections evacuated. Under the reduced pressure the melted agar bubbled and gave up its dissolved gases. After successively evacuating and filling the jar with the gas three times, both lines to the culture flask were tightly clamped and the connections broken. The rubber stopper was shellaced and the bottle placed on its side until the agar had hardened into a thin, even layer. It was then incubated at 37°C.

When using O_2 or CO_2 the culture bottle was always washed out three times with H_2 before the other gases were introduced. Controls were prepared in an identical manner but air was used to fill the bottles instead of the various gases.

EXPERIMENTS WITH ATMOSPHERES OF PURE HYDROGEN

A series of experiments were done by replacing the atmosphere in the culture flasks with H_2, purified as already described. Meat-extract agars of pH 5.9 and 7.2 respectively were used. Inoculation was with a suspension of spores of *Bacillus subtilis*. After 4 days incubation at 37°C. there was no evidence of growth.[1] Abundant growth appeared in sealed control cultures incubated in air, and within 24 hours in the experimental cultures after air had been admitted, thus proving that the spores remained dormant through the incubation period in the atmosphere of H_2. That anaerobic conditions existed during H_2 exposure was indicated by testing some uninoculated flasks in which sterile solutions of glucose and methylene blue had been added to the agar. The dye was decolorized within 24 hours and remained colorless until the bottles were opened.

[1] Growth was recorded as positive in these experiments when macroscopic colonies appeared. This involved the germination of spores and subsequent multiplication of the vegetative cells.

These results were expected, of course, since *Bacillus subtilis* is considered to be an obligatory aerobe.

EXPERIMENTS WITH ATMOSPHERES OF PURE CARBON-DIOXIDE AND WITH MIXTURES OF CARBON-DIOXIDE AND HYDROGEN

Twelve culture flasks (eight at pH 7.6 and four at pH 5.9) were inoculated with spores and incubated in 100 per cent CO_2. Two cultures (pH 5.9) showed some evidence of growth, due probably to leakage. No growth appeared in the others after 2 to 8 days incubation. Seven of the flasks were then opened to the air. Growth appeared promptly. To the other three flasks 10 to 18 cc. of air were admitted after the 2nd, 3rd, and 4th days of incubation, respectively. Growth promptly appeared in two of these flasks. The third was opened to the air 24 hours later and this was followed by prompt growth. In later experiments a buffered medium, pH 7.8, was used to counteract the increased acidity due to the saturation of the medium with CO_2. Methyl red, added to such media, did not change color appreciably when it was incubated in atmospheres with a high CO_2 content, thus indicating that the shift in pH is not sufficient to account for the inhibition of growth.

Combinations of CO_2 and H_2 in which the percentage of CO_2 varied from 8 to 80 per cent were tested. In 10 out of 13 trials there was no evidence of growth (in the other 3 cases development occurred, presumably because of air leakage). Of the 10 cultures in which growth was completely inhibited, eight grew promptly when air was admitted on the 4th, 5th, or 6th day, and two did not when opened on the 6th and 7th day.

In five instances cultures were incubated for three days in mixtures of CO_2 and H_2. No growth appeared. On the 4th day quantities of air varying from 14 to 20 cc. were introduced, and the flasks were again sealed. In four instances growth appeared on the following day. In six experiments the atmosphere consisted of an admixture of 4 per cent of air with CO_2. All of these cultures, except one, grew promptly but meagerly. After the first day of incubation there was no further development until air was admitted on the 5th or 6th day. Luxuriant growth then promptly appeared.

EXPERIMENTS WITH ATMOSPHERES OF PURE OXYGEN AND OF VARYING CONCENTRATIONS OF OXYGEN MIXED WITH HYDROGEN AND CARBON-DIOXIDE RESPECTIVELY

Five experiments were done using an atmosphere of pure O_2. Spore suspensions were used for inocula. All cultures developed promptly and luxuriantly. Mixtures of O_2 and CO_2 brought out the fact that in all combinations in which O_2 was present to the extent of 47 per cent, or higher, the growth obtained was as good as that of the aerobic controls. Good growths, only slightly inferior to those of the controls, were obtained in concentrations of 30 to 47 per cent. In concentrations of 5 and 20 per cent only meager growths resulted. After air had been admitted on the 5th day there was a prompt increase in growth in those cultures where inhibition had been evident.

Cultures incubated in atmospheres of O_2 mixed with H_2 showed normal growth in concentrations of O_2 from 100 to 28 per cent. In concentrations of 11 and 9 per cent there was considerable inhibition. It would thus appear that the concentration of O_2 found in the atmosphere (about 20 per cent) is close to the minimum that will support normal growth of this organism.

THE EFFECT OF VARIOUS GASES ON VEGETATIVE CELLS OF B. SUBTILIS

Another series of experiments was done with vegetative cells as the inoculum.

Of 8 trials in 100 per cent H_2, seven failed to produce visible growth while one showed very slight growth probably due to leakage. The incubation period in 6 of the trials was from 13 to 18 days. When the cultures were incubated after that time with free access to air, growth appeared in four flasks, in two which had been adjusted at pH 5.9 and two at pH 7.8. Cultures held under the same conditions, but inoculated with spores developed on subsequent aerobic incubation.

In three instances where the atmosphere was composed of 21 per cent CO_2 and 79 per cent H_2, vegetative cells produced no visible growth after 4 and 5 days' incubation. A luxuriant growth resulted on subsequent aerobic incubation.

In two trials with an atmosphere composed of 21 per cent CO_2 and 79 per cent O_2 and in one trial with an atmosphere composed of 10 per cent CO_2 and 90 per cent O_2 the vegetative cells produced prompt and profuse growth. In 100 per cent O_2 the vegetative cells likewise produced excellent growth.

DISCUSSION

It would be natural to expect a complete inhibition of growth of *B. subtilis* in 100 per cent H_2 since the organism is considered an obligate aerobe. Not until extreme precautions were taken to remove dissolved gases from the medium was complete inhibition obtained. Indeed it is shown in the experiments with CO_2 that as little as 4 per cent by volume of air (the equivalent of 0.8 per cent O_2) was enough to initiate growth.

The definite inhibitory action of CO_2 on bacteria belonging to the genus *Bacillus* and the genus *Mycobacterium* was pointed out by Coyne (1933). The fact that Coyne was able to obtain growth of most of his aerobic spore bearers in 100 per cent CO_2 is not in agreement with the results reported in this paper. Although he did not use *B. subtilis* in his experiments it seems quite probable that had all precautions been taken to remove dissolved gases from the culture medium, complete inhibition would have resulted in 100 per cent CO_2. Not until this was done was I able to inhibit *B. subtilis* in 100 per cent H_2 or in 100 per cent CO_2. Coyne showed conclusively that the acidity resulting from an atmosphere of CO_2 was not responsible for the inhibition of bacterial growth. He interprets his findings as due to the specific inhibitory effect of CO_2 on the bacteria. Although sufficient evidence is not presented in my data to definitely confirm the inhibitory action of CO_2, my results seem to show that CO_2 in concentrations of 50 per cent or more has some inhibitory action on *B. subtilis*. However, my results show definitely that a very slight amount of O_2 will permit of macroscopic growth in atmospheres containing as much as 99 per cent CO_2. In other words, the limiting factor in the germination of *B. subtilis* spores and subsequent growth of the vegetative cells is not the increased amount of CO_2 in the atmosphere, but the decreased O_2 supply.

According to Rockwell, (1921, 1923), CO_2 is indispensable for the growth of aerobic organisms. I have not found this to be so. Undoubtedly all living organisms produce CO_2 as a result of their metabolic activities, and since in my experiments this gas was not absorbed, some probably accumulated in all cultures that grew. The critical point, however, in such cultures is at the time growth is initiated and at that time there could not have been any appreciable amount of CO_2 in the oxygen and oxygen-hydrogen atmospheres which supported active growth. All indications point to O_2 as the one gas essential to the organism. Soule (1928) found, as I did, that *B. subtilis* grew well in an atmosphere consisting wholly of O_2. He also found that this organism grew on plates in which NaOH was held to absorb the CO_2. Recently Knaysi and Dutky (1934) concluded: "... that the limiting factor in the growth of *Bacillus megatherium* in vacuum is the O_2 content and not the oxidation-reduction potential of the culture medium."

On the other hand, Valley and Rettger (1926) obtained no growth of several aerobic spore-bearing bacilli including *B. subtilis*, when CO_2 was removed from the atmosphere. They incubated their cultures in CO_2-free air in plates inverted over dishes of calcium hydrate paste. Novy and his co-workers (1925) state that although aerobic organisms require O_2 there was reason to believe that without CO_2 some organisms could not fully utilize the O_2.

In discussing this subject, Soule (In Jordan & Falk, 1929) reaches the conclusion that it is the intracellular CO_2 that is important, and if this is removed by exposure of the organisms to alkali, inhibition of growth and even death results. This conclusion was based upon his finding that, whereas the organisms multiplied rapidly on agar surfaces in atmospheres free of all traces of CO_2 they were inhibited when alkali or other CO_2 absorbents were included in the incubating chamber. Organisms developing in a fluid medium were not inhibited by such absorbents, probably because their effects did not actually reach the organisms.

In this work it was found that the organisms grew luxuriantly and there was no evidence of injury either to spores or vegetative

forms when cultures were incubated in atmospheres high in O_2, or even in 100 per cent O_2. This is in agreement with the findings of Moore and Williams (1911) and Karsner *et al.* (1923).

CONCLUSIONS

1. In an atmosphere of 100 per cent H_2, spores of *Bacillus subtilis* do not germinate and the vegetative cells do not multiply. The spores remain viable and germinate when air is admitted while vegetative cells more often fail to grow.

2. In an atmosphere of 100 per cent CO_2, spores of *Bacillus subtilis* do not germinate. The spores remain viable and germinate when air is admitted.

3. In varying mixtures of H_2 and CO_2 no growth occurs in spore-inoculated media. No growth of vegetative cells results in mixtures of 79 per cent H_2 and 21 per cent CO_2.

4. In an atmosphere of CO_2 the introduction of as little as 4 per cent air permits the germination of spores and subsequent multiplication of vegetative cells to produce a visible growth.

5. An atmosphere of 100 per cent O_2 is neither toxic nor inhibitory to the spores or vegetative cells.

6. Germination of spores and multiplication of vegetative cells takes place in mixtures of CO_2 and O_2. The amount of growth resulting from spore-seeded media varies within certain limits with the supply of available free O_2. When the proportion of O_2 in these mixtures is as great as, or greater than, the amount present in the air, the extent of the resulting growths is approximately as great as that in air. Lesser amounts of O_2 give proportionately poorer growths. As little as 0.8 per cent of oxygen permits of macroscopically visible growth.

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CULTURAL REQUIREMENTS OF THE FOWL-CORYZA BACILLUS

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DeBlieck (1932), Nelson (1933), Eliot and Lewis (1934), Delaplane and Stuart (1934) and Schalm and Beach (1934) have reported the isolation from chickens affected with infectious coryza on farms in Holland, New Jersey, Maryland, Rhode Island and California, respectively, of a hemophilic bacillus with which a coryza of short duration can regularly be produced in susceptible chickens. DeBlieck pointed out its similarity to the influenza bacillus of man, both in its morphology and cultural requirements, and proposed the name *Bacillus haemoglobinophilus-coryzae-gallinarum*. Eliot and Lewis proposed the binomial *Hemophilus gallinarum*. Delaplane has expressed his willingness to accept this latter name, but Nelson (1935) expressed his opinion that there is as yet insufficient knowledge concerning this organism to warrant such a definite classification.

The fowl-coryza bacillus is a small, Gram-negative, non-motile rod which is highly pleomorphic and frequently develops into irregularly staining filaments, often of remarkable length. On two occasions branching of these filaments was observed. The bacillus shows a marked tendency to stain more heavily at the poles than in the center, and frequently a deeply staining granule is observed at each pole, while the remaining portion takes the stain less and less as the center is approached. In cultures more than twenty-four to forty-eight hours old, the bacteria usually are swollen, stain faintly and finally become fragmented so that only shapeless masses are found. This degeneration has been observed in cultures only eighteen hours old, but this is exceptional. The phenomenon has been noted on all media on which growth has been obtained.

Davis (1917, 1921), Thjötta and Avery (1921), Fildes (1921) (1922), and Rivers and Poole (1921) have shown that *Hemophilus influenzae*, the type species of the genus *Hemophilus*, requires for its growth two accessory factors in addition to a nutrient medium. These factors were likened to vitamins A and B by Davis, and later were designated as X and V factors by Thjötta and Avery. The X factor is heat stable, the V factor resists boiling for five minutes but is destroyed by autoclaving. Hemoglobin is the principal source of the X factor, although it is present to some extent in certain vegetable tissues, especially potatoes. The V factor is more widely distributed in nature, being produced by nearly all species of bacteria and present in perhaps all fresh animal and vegetable tissues. Blood is an excellent source of both factors, the X factor being confined to the red blood cells and the V factor present in both the red cells and the serum.

CULTURE MEDIA AND SOURCES OF X AND V FACTORS[1]

Boiled blood agar. (Levinthal's medium, 1918.) This contains both the X and the V factors and was prepared as follows: Defibrinated blood was added to melted nutrient agar in sufficient quantity to produce a concentration of 8 per cent. The mixture was then boiled for several minutes and filtered through sufficient thicknesses of sterile cheesecloth to remove the coagulated particles. The cultures used in the experiments reported in this paper had been grown for many generations on this medium, which, when properly prepared, is transparent and free of blood pigments. Since much of the hemoglobin is removed in the preparation of boiled blood agar, it should contain less of the X factor than fresh blood agar consisting of 8 per cent defibrinated blood in an agar base made with beef extract. This is an important consideration, for Davis (1907) has shown that the amount of X factor required for growth of hemophilic bacilli is very small, and Thjötta and Avery (1921) that enough of the X factor to support growth of a hemophilic organism may be carried over in making transplants from a medium containing an abundance of this factor to one deficient in it.

[1] Horse blood used exclusively. All media adjusted to pH 7.4.

On boiled blood agar, the colonies are convex, smooth, clear to slightly opaque and glistening, and vary in diameter from 0.05 mm. or less to 0.6 mm., while on fresh blood agar, the colonies are smaller, rarely exceeding 0.3 mm. in diameter, less convex, more opaque, dull to slightly glistening and show a marked tendency to undergo rough transformation. While the reason for the difference in the size and character of the colonies of the organism on the two media is not clear, it might be that fresh blood contains a factor which has an inhibiting influence on the growth of the organism and which is destroyed by heat, and, therefore, not present in boiled blood agar.

Autoclaved blood agar. This contains only the X factor, and was prepared in the same manner as boiled blood agar except that it was autoclaved at 15 pounds for thirty minutes.

Autoclaved blood extract. This contains only the X factor, and was prepared according to the method of Rivers and Poole (1921) as follows: A clot from 500 cc. of blood was infused with 200 cc. of 0.85 per cent saline solution, boiled, filtered through cheesecloth, tubed and autoclaved at 15 pounds for thirty minutes.

Serum from defibrinated blood. This contains both the X and the V factors. Freshly-drawn blood was defibrinated, centrifuged at a high speed for twenty minutes, and the supernatant serum removed to sterile test tubes and stored in the refrigerator. The defibrination procedure breaks up some of the red cells and thus liberates some of the X factor into the serum.

Serum from clotted blood. This contains the V factor only. Blood was allowed to clot in sterile tubes, and then centrifuged at a high speed for twenty minutes. The supernatant serum was transferred to sterile tubes and stored in the refrigerator.

Washed red blood cells. This contains both the X and the V factors. The blood cells remaining in the centrifuge tube, after removal of the serum in preparation of serum from defibrinated blood, were washed three times by resuspending them in saline and centrifuging. After the third washing, the cells were suspended in a volume of saline equal to that of the serum, and stored in the refrigerator.

GROWTH OF THE FOWL-CORYZA BACILLUS ON BOILED OR FRESH BLOOD AGAR PLATES OR SLANTS INCUBATED AEROBICALLY, IN AN EXCESS OF CARBON DIOXIDE, OR WITH THE PLATES AND TUBES SEALED WITH CLAY OR PARAFFIN

DeBlieck, Delaplane and Stuart, and Eliot and Lewis have reported no difficulty in obtaining growth of the fowl-coryza bacillus under aerobic conditions. Nelson, however, stated that he was unable to grow his organism on open plates but succeeded very well when the plates were sealed with clay. Schalm and Beach reported failure to cultivate the organism either aerobically or on sealed plates, but obtained good growth when the cultures were placed in an atmosphere of 10 per cent carbon dioxide. The writers have since investigated this problem further with cultures obtained from Nelson and from Delaplane and with California strains of the fowl-coryza bacillus. Regardless of source or strain, the organism colonized not at all or very meagerly on open plates or slants incubated aerobically, but satisfactory growth was obtained on sealed plates or slants or on unsealed plates or slants in sealed jars containing ordinary air or 10 per cent carbon dioxide. The growth of the bacillus on the sealed plates or slants might be explained on the basis of Rockwell's (1927, 1928) theory that carbon dioxide is the principal source of the carbon required by bacteria for multiplication; that the carbon dioxide produced through the metabolic activities of bacteria may be used by them as a source of carbon; and that when the carbon dioxide produced through metabolic activity is not allowed to escape from culture plates or tubes the concentration of it in the contained air would be increased and the growth of certain species of bacteria thereby favored. In the experiments presented herein all cultures were incubated in sealed jars containing 10 per cent carbon dioxide.

CULTURES USED IN THE EXPERIMENTS

Twelve different cultures of the fowl-coryza bacillus, representing a New Jersey strain, a Rhode Island strain and six California strains, were employed. These cultures varied in their period of cultivation on artificial media which ranged from 4 to 157

transfers made every 4 days. All cultures were maintained on boiled blood agar slants with broth at the base. The growth was washed from the slant into the broth and a 2 mm. loopful was transferred to the test medium. In one experiment, single colonies were transferred from a boiled blood agar plate to the test medium.

TABLE 1

Growth of the fowl-coryza bacillus on a medium containing only the X factor compared with its growth on a medium containing both the X and the V factors

STRAIN	FOUR-DAY TRANSFER NUMBER	GROWTH AFTER SEVENTY-TWO HOURS INCUBATION*		
		Boiled blood agar slant (X and V factors)	Autoclaved blood agar slant plus 1 cc. horse serum (X and V factors)	Autoclaved blood agar slant plus 1 cc. plain broth (X factor)
Calif. 1	114th	+++	++	0
Calif. 3	70th	+++	++	0
Calif. 1	54th	+++	++	0
Calif. 4	54th	+++	++	0
Rhode Island N 37	20th	+++	+	0
Calif. 4	19th	+++	++	0
Rhode Island N 37	18th	+++	++	0
Calif. 6	6th	+++	++	0

*+++ = Good growth.
++ = Fair growth.
+ = Slight growth.
0 = No growth.

GROWTH OF THE FOWL-CORYZA BACILLUS ON A MEDIUM DEVOID OF BOTH X AND V FACTORS

Seven cultures, representing 1 New Jersey, 1 Rhode Island and 4 California strains, were employed. Transfers were made to plain nutrient agar which contained neither of the two factors and to boiled blood agar slants which supplied both factors. These cultures were incubated at 37°C. for ninety-six hours. The plain agar failed to support growth of any of the cultures, but a very good growth of all cultures was obtained on the boiled blood agar controls.

GROWTH OF THE FOWL-CORYZA BACILLUS ON A MEDIUM CONTAINING X FACTOR ONLY

The cultures and media shown in table 1 were employed. It is seen that the bacillus failed to grow on autoclaved blood agar

TABLE 2

Growth of the fowl-coryza bacillus in a fluid medium devoid of the X and V factors, or containing only the X or only the V factor, or containing a combination of the X and V factors

STRAIN	FOUR-DAY TRANSFER NUMBER	GROWTH AFTER SEVENTY-TWO HOURS INCUBATION*					
		5.0 cc. plain broth	4.5 cc. plain broth plus 0.5 cc. autoclaved blood extract (X factor)	4.5 cc. plain broth plus 0.5 cc. serum from clotted blood (V factor)	5.0 cc. plain broth plus 2 drops of washed red cells (X and V factors)	4.5 cc. plain broth plus 0.5 cc. serum from defibrinated blood (X and V factors)	4.0 cc. plain broth plus 0.5 cc. autoclaved blood extract plus 0.5 cc. serum from clotted blood (X and V factors)
Calif. 3	104th	0	0	0	+++	+++	+++
Calif. 6	54th	0	0	0	+	++	+++
Rhode Island N 37	50th	0	0	0	+	++	++
New Jersey B 5504	40th	0	0	0	+	++	++
Calif. 5	4th	0	0	0	0	+	+

* The relative degree of growth was ascertained by streaking a 2 mm. loopful of each culture on a boiled blood agar plate which was incubated at 37°C. for 48 hours.

+++ = 100 or more colonies.
++ = 50 to 100 colonies.
+ = 25 to 50 colonies.
0 = No growth.

plus broth which contained the X factor only, but when, instead of broth, serum, to supply the V factor, was added, colonies developed which were of microscopic size, flat and dull, and largest near the serum at the base of the slant. On the boiled blood agar controls, the colonies were smooth, convex, slightly opaque and glistening, and varied from 0.05 mm. or less to 0.4 mm. in diameter.

GROWTH OF THE FOWL-CORYZA BACILLUS IN A FLUID MEDIUM CONTAINING X FACTOR ONLY, V FACTOR ONLY, AND BOTH FACTORS

The cultures and media to supply the X and V factors alone or in combination, which were used in this experiment, are given in table 2.

No macroscopic change occurred in any of the culture tubes; so, to obtain evidence of bacterial growth, a loopful of each culture was streaked on a boiled blood agar plate which was incubated at 37°C. for forty-eight hours. All cultures failed to develop in plain broth or in plain broth plus autoclaved blood extract (X factor only) or in plain broth plus serum from clotted blood (V factor) but in the media containing both X and V factors, growth of all strains was obtained. It is seen, however, that the medium containing washed red cells supported growth less effectively than that containing serum from defibrinated blood or that containing serum from clotted blood plus autoclaved blood extract, and that in the case of a recently isolated California strain 5, no growth occurred in the medium containing washed red blood cells. A possible explanation of this difference is that much of the X and the V factors remained within the cells instead of being dispersed through the broth. The comparatively weak growth obtained in any instance indicates that none of the media were favorable to the organism, but the results, nevertheless, show that both the X and the V factors must be present before the bacillus can develop.

GROWTH OF THE FOWL-CORYZA BACILLUS ON PLATES CONTAINING X FACTOR FROM BLOOD AND V FACTOR FROM A BACTERIAL COLONY

The sources of the X and the V factors and the cultures employed in this experiment are shown in table 3.

A single colony of the fowl-coryza bacillus was streaked on each plate of test medium, and, to provide the V factor to some of them, the center of the seeded area was inoculated with *Serratia marcescens*.

No growth appeared on the media containing the X or the V

factor alone but colonies invariably developed when both of these factors were present. The colonies of the fowl-coryza bacillus on the autoclaved blood agar plates having a *Serratia marcescens* colony varied in size and shape according to their distance from this V-factor-producing colony, those within 0.5 cm. of it being smooth, convex and clear to slightly opaque and from 0.15 to 0.25 mm. in diameter; those at a distance of 1 cm. being of the same appearance but not exceeding 0.1 mm. in diameter, and those from 1.5 to 2.0 cm. distant being microscopic in size, flat, and often dull and rough.

TABLE 3

Growth of the fowl-coryza bacillus on plates containing X factor from blood and V factor from a bacterial colony

STRAIN	FOUR-DAY TRANSFER NUMBER	GROWTH AFTER FORTY-EIGHT HOURS INCUBATION*			
		Boiled blood agar plate (X and V factors)	Autoclaved blood agar plate plus 1 *S. marcescens* colony (X and V factors)	Autoclaved blood agar plate (X factor)	Plain agar plate plus 1 *S. marcescens* colony (V factor)
Calif. 1	112th	+++	+	0	0
Rhode Island N 37	79th	+++	+	0	0
Calif. 6	77th	+++	+	0	0
New Jersey B 5504	65th	+++	+	0	0
Calif. 1	24th	+++	+	0	0
Calif. 8	14th	+++	+	0	0

*+++ = Good growth over entire seeded area.
+ = Growth only in the immediate vicinity of the *S. marcescens* colony.
0 = No growth.

This experiment clearly demonstrates the necessity of both the X and the V factors for growth of the fowl-coryza bacillus on an artificial medium. It also shows, as the previous experiments have done, that even after prolonged cultivation on culture media, the organism still required the presence of the two factors for growth.

DISCUSSION AND SUMMARY

Experiments are reported in which an effort was made to determine whether the fowl-coryza bacillus requires the presence of

both the X and the V factors for growth on or in an artificial medium. Twelve different cultures, representing 1 New Jersey, 1 Rhode Island and 6 California strains, were studied. These cultures varied in their period of cultivation on artificial media for from 4 to 157 transfers made every four days.

It was found that, regardless of strain or age, all cultures of the fowl-coryza bacillus required the presence of both the X and the V factors for growth on or in an artificial medium. On this basis, the fowl-coryza bacillus is to be classed in the genus *Hemophilus*. The name *Bacillus haemoglobinophilus-coryzae-gallinarum*, suggested by deBlieck, describes the organism satisfactorily, but, because of its length, is awkward and contrary to the rules of nomenclature.[2] The binomial *Hemophilus gallinarum*, proposed by Eliot and Lewis, seems better suited for this organism.

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[2] Bergey's Manual of Determinative Bacteriology, 1934, 4th ed., p. 24, line 35.

A COMPARISON OF MEDIA FOR THE DETECTION OF ESCHERICHIA-AEROBACTER

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In a recent contribution Noble and White (1935) compared fuchsin lactose, formate ricinoleate, crystal violet, brilliant-green bile and Difco lactose broth with standard lactose broth and found that all were satisfactory by conventional inoculation. However, by the Butterfield dilution method with results expressed by the Hoskins Most Probable Number, using a single strain of *Escherichia coli* only formate ricinoleate and Difco lactose broth were good enough for further comparative study.

Unpublished results obtained by us using a conventional method and an inoculum of 100, 10 or 1 organism per tube showed that all of the liquid media tested were about equally effective in showing gas production with the strains of *Escherichia-Aerobacter* used. Accordingly a comparative test by the Butterfield dilution method was carried out.

The media used included standard lactose (A. P. H. A., 1933), crystal violet (Salle, 1930), buffered lactose (Thompson, 1927), and fuchsin (Ritter, 1932), broth, all of which were prepared according to the Standard Methods of Water Analysis (A. P. H. A., 1933) or the directions of the respective authors. Other media used were Difco dehydrated brilliant-green bile (Dunham, McCrady and Jordan, 1925), formate ricinoleate (Stark and England, 1933) and methylene-blue brom-cresol purple media (Dominick and Lauter, 1929), the latter having been prepared according to the revision before June, 1933, except in the tests with the intermediate strain C and *Aerobacter aerogenes* strain C.

[1] This article is based on part of the experimental work for the Master of Science thesis, University of Maryland, by the junior author.

All media were stored in a refrigerator and kept not more than two weeks before use.

The organisms used included laboratory cultures of *Escherichia coli*, of *Aerobacter aerogenes* and of an intermediate strain which were used in the previous tests already mentioned. Furthermore recently isolated strains of *Escherichia coli*, of *Aerobacter aerogenes* and of an intermediate strain were secured from the Referee for bacteriological methods for water of the A. P. H. A. Standard Methods Committee.[2] These cultures were obtained from water in the comparative productivity test (A. P. H. A., 1935) for the coli-aerogenes group carried on by that committee. Hereafter, the culture strains will be designated as C, and the others as L or laboratory strains. Since *Aerobacter aerogenes* L681-1 gave questionable reactions with the methyl red and Voges-Proskauer reactions, strain L8 was substituted after the first set of media had been tested.

The partially confirmed test (Endo's medium) and completed tests were made with a lactose broth culture in order to check the organisms. Bacto dehydrated Methyl-Red Voges-Proskauer medium was used in classifying the different pure cultures of the *Escherichia-Aerobacter* group used, tests being made after 48 hours. Difco tryptophane broth was used for the indol test which was made after four days with Ehrlich's reagent on the cotton plug (acid paradimethylamidobenzaldehyde and saturated persulphate). Bacto dehydrated Simmon's citrate agar (Simmons, 1926) was used in the identification, the readings being taken after 24- and 48-hour periods. Confirmatory tests according to the English procedure, using lactose peptone broth and brom-cresol purple milk, were also made on the laboratory strains. The reactions obtained with these cultures are recorded in table 1.

The stock suspension was prepared by transferring a 0.5 ml. portion of a 24-hour lactose broth culture (incubated at 37°C.) to a bottle containing 99 ml. of the Butterfield (1933) dilution water. Its approximate titer was determined by inoculating triplicate tubes of lactose broth with 1:1,000, 1:10,000, 1:100,000, 1:1,000,-

[2] We are indebted to this Committee for a copy of the directions they distributed which were followed in this work wherever possible.

TABLE 1

Reactions of experimental cultures

(Tests made at least in duplicate)

ORGANISM	STRAIN	ENDO	INDOL	METHYL RED	VOGES-PROSKAUER	CITRATE
E. coli	L	+	+	+	−	−
E. coli	C	+	+	+	−	−
A. aerogenes	L 681-1	+	−	±	±	+
A. aerogenes	L 8	±	−	−	+	+
A. aerogenes	C	−	−	−	+	+
Intermediate	L 118-1	±	−	+	−	+
Intermediate	C	+	−	+	−	+

+ represents a positive reaction, growth, or on Endo's agar, a metallic sheen.

TABLE 2

Titer of stock suspension determined by triplicate inoculation of various dilutions

Number of gas-producing tubes recorded

ORGANISM	STRAIN	EXPERIMENT	DILUTION											
			1:1,000		1:10,000		1:100,000		1:1,000,000		1:10,000,000		1:100,000,000	
Hours incubation			24	48	24	48	24	48	24	48	24	48	24	48
E. coli	L	1	3	3	3	3	3	3	1	1	0	0	0	0
E. coli	L	2	3	3	3	3	3	3	0	0	0	0	0	0
E. coli	C	1	3	3	3	3	3	3	0	0	0	0	0	0
A. aerogenes	L	1	3	3	3	3	3	3	2	2	2	2	0	0
A. aerogenes	C	1	3	3	3	3	3	3	3	3	0	0	0	0
A. aerogenes	C	2	3	3	1	2*	0	3	0	1	0	0	0	0
Intermediate	L	1	3	3	3	3	3	3	1	1	0	0	0	0
Intermediate	C	1	3	3	3	3	3	3	0	0	0	0	0	0

* The one tube which did not produce gas in 48 hours showed gas in less than three days.

000, 1:10,000,000 and 1:100,000,000 dilutions made also in the Butterfield dilution water. These 18 tubes were read after 24 hours' incubation at 37°C. and the highest dilution in which all three

tubes showed gas was taken as the lowest dilution in the comparative productivity test. The titer of the cultures used is reported in table 2.

The suspension was used before 48 hours had elapsed after its preparation. Fifteen tubes of each of three dilutions were used for each medium, and three trial media were tested at one time with the lactose broth, making a total of 180 tubes inoculated at a time, or, in the two series, 360 tubes inoculated with one organism.

The stock suspensions were shaken vigorously for two minutes, and then dilutions were made into the required number of 99 ml. blanks (usually three). Two dilutions were secured from one bottle. For example, from the second bottle a 1 ml. inoculum gave a 1:10,000 dilution, while a 0.1 ml. inoculum gave a 1:100,000 dilution. Graduated 1 ml. and 10 ml. pipettes were used for the inoculations. *The different tubes of media were inoculated in turn*, for example: (1) lactose, (2) brilliant-green bile, (3) crystal violet, (4) formate ricinoleate; and then repeated in this order to eliminate discrepant results due to the time factor. The dilution bottle was shaken vigorously every time a pipette was reintroduced. Care was taken to place the 0.1 ml. portion in the medium and not on the side of the tube. The planted tubes were incubated at 37°C. and readings were made after 24 and 48 hours. The presence of any quantity of gas was recorded as positive and its absence as negative. The tests were repeated with two of the organisms (experiment *2*) since at first these tubes were nearly all negative. The data obtained are recorded in table 3.

In determining the titer of the stock suspensions (table 2) the results were fairly regular except in one case with *Aerobacter aerogenes*. This organism fermented lactose very slowly and seemed to die easily in the buffer of the suspension liquid. After 24 hours, the reading of the stock titer determination was inadequate to give results of any value. In the two experiments in which this organism was used (table 3) of 360 tubes inoculated, only 27 were positive the first time and 50 in the next experiment. The limit set by the Committee (A. P. H. A., 1935) was at least

TABLE 3A

Comparison of gas production by Butterfield dilution method with various fermentation media for the Escherichia-Aerobacter group

Results indicate number of positive tubes of 15 inoculated

ORGANISM	STRAIN	EXPERIMENT	DILUTION	SERIES I							
				Standard lactose		Brilliant green bile		Salle crystal violet		Formate-ricinoleate	
Hours incubation				24	48	24	48	24	48	24	48
E. coli	L	1	A	12	13	0	0	0	1	0	0
			B	2	2	1	1	0	0	0	0
			C	1	1	0	0	0	0	0	0
E. coli	L	2	A	15	15	15	15	0	8	3	3
			B	4	4	6	6	0	2	0	0
			C	0	0	0	0	0	0	0	0
E. coli	C	1	A	15	15	2	2	1	9	2	2
			B	7	7	2	2	1	3	0	0
			C	0	0	0	0	0	0	0	0
A. aerogenes	L	1	A	9	9	6	6	2*	2*	2	2
			B	1	1	1	1	0*	0*	1	1
			C	0	0	0	0	0*	0*	0	0
A. aerogenes	C	1	A	1	3	3	3	3	5	0	0
			B	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0
A. aerogenes	C	2	A	1	5	3	5	0	2	0	0
			B	0	0	0	0	0	1	0	0
			C	0	0	0	0	0	0	0	0
Intermediate	L	1	A	11	11	12	12	6	9	7	9
			B	0	1	0	1	0	0	1	2
			C	2	2	1	1	0	0	0	0
Intermediate	C	1	A	8	8	7	7	4	6	5	5
			B	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0
Total positive				89	97	59	62	19	50	21	24

* These were inoculated at the same time as the other series of standard lactose, and should be compared with the other series' standard (the total reported being corrected for this).

TABLE 3B

Comparison of gas production by Butterfield dilution method with various fermentation media for the Escherichia-Aerobacter group

Results indicate number of positive tubes of 15 inoculated

ORGANISM	STRAIN	EXPERIMENT	DILUTION	SERIES II							
				Standard lactose		Dominick-Lauter		Buffered lactose		Ritter fuchsin	
Hours incubated				24	48	24	48	24	48	24	48
E. coli	L	1	A	1	1	1	1	2	2	0	2
			B	0	0	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0
E. coli	L	2	A	15	15	14	14	15	15	15	15
			B	6	8	4	4	4	4	6	7
			C	1	1	1	1	1	1	1	1
E. coli	C	1	A	11	11	14	14	15	15	13	15
			B	5	6	3	3	5	5	1	1
			C	1	1	0	0	0	1	0	0
A. aerogenes	L	1	A	5	5	4	4	7*	7*	4	4
			B	0	0	1	1	0*	0*	0	0
			C	0	0	0	0	0*	0*	0	0
A. aerogenes	C	1	A	0	1	0	3	2	2	0	0
			B	0	1	0	0	0	0	0	0
			C	0	0	0	0	0	0	0	0
A. aerogenes	C	2	A	1	2	0	1	3	5	5	9
			B	0	1	0	0	1	3	0	2
			C	0	0	0	0	0	0	0	0
Intermediate	L	1	A	9	12	9	13	9	12	8	10
			B	12	14	2	6	6	11	4	9
			C	1	1	1	2	1	3	1	2
Intermediate	C	1	A	4	7	7	7	0	3	5	10
			B	11	11	5	5	5	7	4	7
			C	0	0	0	0	0	0	0	0
Total positive				83	98	66	79	73	93	67	94

* These were inoculated at the same time as the other series of standard lactose, and should be compared with the other series' standard (the total reported being corrected for this).

40 per cent negatives in the highest dilution in the most productive medium, and not less than 20 per cent positives in the lowest dilution in the least productive medium.

In table 3 the main results of the work by the Butterfield dilution method are tabulated. The total of positive tubes for each medium was counted and the standard lactose broth was shown to give more positive tubes in 24- and 48-hour periods than any other medium tested, when working with pure cultures. Buffered lactose and Ritter fuchsin broth were nearly as sensitive. The formate ricinoleate medium gave very poor results. However, this may have been due to the fact that the same pressure was used in autoclaving all of the media at one time, so that the ricinoleate separated out in oil droplets. The directions on the dehydrated medium bottle recommended lower temperature and pressure, but permitted 15 pounds pressure as an alternative. Since excellent results were obtained by Noble and White (1935) who worked with the Stark-England medium, it may be that the lower pressure and temperature only should be specified. Salle crystal-violet medium inhibited about 50 per cent of the organisms (using standard lactose results as 100 per cent) with the small inoculum used.

The discrepancy between the amounts of gas produced in the brilliant green bile medium by the laboratory strain of *Escherichia coli* in the two experiments performed with this organism is unexplainable. Approximately the same results were obtained with the C strains. The rate of death of the organism in the buffered dilution water was demonstrated by the two experiments with the laboratory strain of *Escherichia coli*. There was a 24-hour interval between the inoculation of the first series of trial media and the second series in L-1, reflected in the control lactose broth by a radical reduction in the number of tubes giving gas, while there was an interval of only about an hour in L-2 and little decline in numbers. Allowance was made thereafter for the death-rate by using the next lowest dilution if a 24-hour period had elapsed. Even this was not sufficient to compensate for the decrease with *Aerobacter aerogenes* as may be seen by a comparison of tables 2 and 3.

Three groups of inconsistent readings were noted, two occurring with the laboratory strain of an intermediate in standard lactose and in buffered lactose broth, and one occurring with the C strain of intermediate in standard lactose broth. More positive results were obtained with the mean dilution than with the low dilution in all 3 cases. A chance distribution of the organism may have caused this phenomenon. The amount of inoculum in all cases was 1 ml. in the low dilution and in the mean, 0.1 ml.

SUMMARY

1. The media tested by the Butterfield dilution method were ranked in order of decreasing sensitivity, as standard lactose, buffered lactose, fuchsin, methylene-blue brom-cresol purple, brilliant-green bile, crystal violet and formate ricinoleate broth.

2. Buffered lactose, Ritter fuchsin and Dominick-Lauter mediums were about equal in sensitivity to standard lactose with the pure cultures of *Escherichia coli* used but were not superior to standard lactose. This was also true to a lesser extent with the cultures of *Aerobacter aerogenes* and of the intermediates used.

3. Brilliant green bile was equal to standard lactose in sensitivity with *Aerobacter aerogenes* and intermediate strains but not uniformly so with the strains of *Escherichia coli*.

4. The crystal violet and formate ricinoleate mediums as used in these experiments were decidedly inferior to standard lactose in giving gas production with pure cultures of *Escherichia coli*. This was true to a lesser extent with cultures of *Aerobacter aerogenes* and to a still lesser extent with the cultures of intermediates used.

5. The 15 pounds pressure used instead of 11 to 13 pounds recommended for sterilization may have accounted for the poor results with the formate ricinoleate medium in the production of gas with members of the *Escherichia-Aerobacter* group.

6. It was necessary to use the next lower decimal dilution than the original titer determined, if the organism had been in the buffered dilution water for 24 hours. It was found advisable either to redetermine the titer or to make allowance for the death of some organisms if more than 24 hours had elapsed.

7. Strains of *Aerobacter aerogenes* uniformly decreased at a more rapid rate than the other organisms in the Butterfield dilution water used.

8. The results of the elaborate procedure of the Butterfield dilution method did allow some differentiation to be made between media but some of these were scarcely clear enough to justify the extra time and media required compared to conventional methods. It is believed that a reduction could be made in the number of tubes used in such comparative tests without altering materially the results obtained.

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THE INFLUENCE OF OXYGEN TENSION ON THE RESPIRATION OF PNEUMOCOCCI (TYPE I)

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Decrease of oxygen tension in the tissues occurs in every stasis of blood and lymph. Determinations of the oxygen tension in fluids taken from an inflamed area in the course of an experimentally-produced sterile inflammation showed, for example, after twenty-four hours an oxygen pressure of 70 mm.Hg., after forty-five hours of 40 mm.Hg. and after sixty-five hours an oxygen pressure of 20 mm.Hg. (Kempner and Peschel, 1930). This fact suggests the question: What influence have relatively low oxygen pressures upon the vital reactions of pathogenic bacteria?

This question can only be answered experimentally by a method which makes it possible to measure quantitatively the energy-yielding processes in bacteria under a constantly maintained equilibrium between reacting cells, culture medium and gas milieu. Some older investigations mentioned in the bacteriologic literature (Kolle and Wassermann, 1929), which were not based on this constantly-maintained equilibrium, are of little value for the solution of this particular problem. They concern themselves more with the diffusion of different gases in different test vessels and culture media than with the effect of these gases upon the reactions of the organism under experimental observation. Then, too, these studies were limited to the acceleration and retardation of bacterial growth, which often is not influenced by factors which inhibit or increase other specific cell reactions, as, for example, lactic or butyric acid fermentation, the forming of hydrogen peroxide and respiration.

The quantitative determination of the metabolic changes in living cells under exact physiological conditions is possible with the manometrical methods of O. Warburg (1927). Experiments carried out by these methods have shown that, with the exception of the nitrogen-fixing *Bacterium azotobacter* (Meyerhof and Schulz, 1932), the respiration of animal body cells, yeast cells and many bacteria is largely independent of variations of oxygen pressure. Even at an oxygen tension of 10^{-5} atm. (0.008 mm.Hg.) the respiration of *Micrococcus candicans*, for instance, was not less than in air (Warburg and Kubowitz, 1929). I have examined *Escherichia coli*, *Pseudomonas pyocyanea*, *Vibrio Metchnikovii*, and *Staphylococcus aureus* at oxygen pressures of 5 to 150 mm.Hg. and have also not found any influence of oxygen pressure on their respiration. In all of these cells the respiration is inhibited by HCN and CO, that is to say, the respiration is catalyzed by a haemin-containing ferment (Warburg, 1931).

An entirely different type of respiratory mechanism was found in the pneumococcus, *Bacillus acidophilus* and *Bacillus acidificans-Delbrueckii*. In these bacteria, which lack catalase, the oxygen consumed in respiration is converted into hydrogen peroxide (McLeod and Gordon, 1922, 1924, 1925; Avery and Morgan, 1924; Bertho and Glueck, 1932; Sevag, 1933; Sevag and Maiweg, 1934). Their respiration is not inhibited by HCN (Meyerhof and Finkle, 1925; Bertho and Glueck, 1932; Sevag, 1933) or by CO (Bertho and Glueck, 1932; Fujita and Kodama, 1934, 1935), that is to say, is not catalyzed by a haemin respiration ferment but by an iron-free ferment, the so-called Warburg yellow oxidation ferment (Warburg and Christian, 1932, 1933), which was demonstrated spectroscopically in *Bacillus acidificans-Delbrueckii* and could be isolated from the cells.

The rate of respiration catalyzed by the yellow ferment itself, separated from the cells, proved to be dependent on oxygen tension: at an oxygen tension of 760 mm.Hg. the oxygen consumption was 4.8 times as much as at a tension of 38 mm.Hg. (Warburg and Christian, 1932). The respiration of the bacteria containing this ferment, however, did not appear to be dependent on variations of oxygen pressure, as shown by studies on *Bacillus*

acidophilus and *B. acidificans-Delbrueckii* (Bertho and Glueck, 1932). Since these determinations extended only to partial pressures of oxygen between 76 and 760 mm.Hg., I have examined the effect of still lower oxygen pressures (15 to 76 mm.Hg.) upon the respiration of these bacteria, but found no difference in the magnitude of respiration either. Likewise I could not discover any effect of oxygen tension (varied between 2 and 20 vol. per cent O_2) upon the respiration of *Clostridium butyricum* which also contains large amounts of the yellow ferment (Warburg and Christian, 1933).

This paper deals with the influence of different oxygen pressures on the respiration and growth of pneumococci.

EXPERIMENTAL

Cultures of pneumococci, type I, two to seventy hours old, grown in broth or on blood agar, were used:[1] In experiments in which very young pneumococci were needed, broth cultures were put into manometer vessels in order to determine exactly the moment at which growth began. The bacteria were suspended in broth (0.5 per cent glucose, pH 7.3) or in a 1:1 mixture of broth and salt solution (0.03 M phosphate, pH 7.3 + 0.131 M NaCl + 0.006 M KCl + 0.03 M glucose) or in salt solution of the above composition plus 0.18 gram magnesium sulphate per liter (Fujita and Kodama, 1934, 1935). To 2 cc. of bacterial suspension 0.2 cc. of a colorless blood (stromata of 500 mgm. erythrocytes) or potato (50 mgm.) catalase solution, were added. In this way the hydrogen peroxide formed in respiration is immediately split into H_2O and O_2 and the bacteria cannot be damaged by an accumulation of hydrogen peroxide.[2] 2 cc. of the solution,

[1] I wish here to repeat my cordial thanks to Dr. W. H. Kelley and Dr. E. N. Scadron for their courtesy in providing me with the cultures.

[2] In order to examine the bacteria under conditions similar to those in the tissues, one has to use media with optimal concentration of catalase, just as one uses buffers to counteract the damaging effect of pH changes. Experiments on the respiration of pneumococci in media which contain no catalase, show a decrease of the respiration rate even after a period of ten to twenty minutes (Finkle, 1931; Sevag, 1933; Sevag and Maiweg, 1934). In the body an accumulation of hydrogen peroxide is not probable because of the large number of erythrocytes present in the inflamed lung.

0.1 to 2 mgm. of pneumococci, weighed after centrifugalizing and drying at 100°C. (Peschel, 1930), were put into manometer vessels. To determine the oxygen consumption the CO_2 produced was absorbed by KOH (0.4 cc. 6 per cent KOH). To determine the respiratory quotient the differences of gas pressures were measured in the presence and absence of KOH; the retention

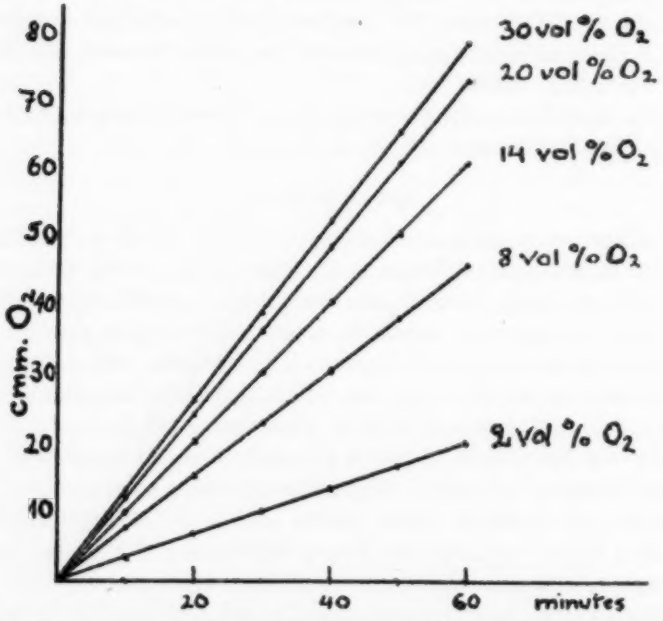

FIG. 1. RESPIRATION OF 0.3 MG. PNEUMOCOCCI AT VARIOUS OXYGEN PRESSURES

The points in the curves show the experimentally determined *total* amount of oxygen consumed in the designated time.

of CO_2 by bacteria and phosphate solution was measured by putting the experimental liquid into the insert well of the manometer vessel and determining the CO_2 which was liberated from a definite amount of bicarbonate, contained in the bottom of the main vessel, on adding lactic acid from the side-arm (Warburg, Kubowitz and Christian, 1931). Mixtures of oxygen-nitrogen or oxygen-argon of varying percentage were prepared over mer-

cury in a gasometer of 2-liters capacity. Fluids and vessels were saturated with these gas mixtures and the metabolism of the bacteria was measured at a temperature of 37°C. The vessels were shaken in the thermostat at such a rate that a further increase of the shaking speed did not change the results.

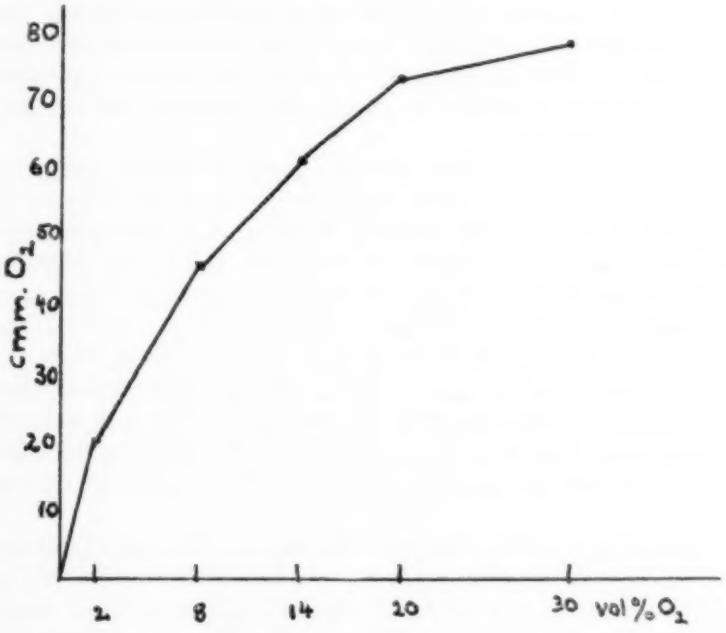

FIG. 2. RATE OF RESPIRATION OF 0.3 MG. PNEUMOCOCCI IN 1 HOUR AS FUNCTION OF OXYGEN PRESSURE

As shown by figures 1 and 2 the respiration of pneumococci depends to a high degree on the oxygen tension. At 20 vol. per cent O_2 (152 mm.Hg.) the oxygen consumption of 0.3 mgm. pneumococci in one hour amounts to 73 cmm.; the amount of oxygen consumed by the same quantity of bacteria in the same time at an oxygen pressure of 2 vol. per cent (15.2 mm.Hg.) is only 20 cmm. At a partial oxygen pressure of 15 mm.Hg. the respiration of pneumococci in comparison with their respiration

under an oxygen pressure of 150 mm.Hg. is lowered by 72 per cent. The respiratory quotient at the various oxygen tensions was found to be between 0.8 and 0.9.

The oxygen partial pressure optimal for the respiration of pneumococci is between 20 and 30 vol. per cent. At higher oxygen pressures the rate of respiration decreases. The same amount of bacteria which consume in one hour at 20 vol. per cent oxygen (152 mm.Hg.) 73 cmm. O_2, consume at 100 vol. per cent O_2 (760 mm.Hg.) only 51 cmm. The rate of respiration in pure oxygen is lowered by 30 per cent compared with the rate of respiration in air.

The dependence of respiration on oxygen pressure is influenced by the same factors which influence the absolute amount of respiration, that is, the manner of cultivation, the suspension medium (pH, concentration of oxidizable substances, $MgSO_4$), and the age of the bacteria. Pneumococci grown on blood agar for forty-five hours showed at an oxygen pressure of 20 vol. per cent a respiration rate twice as high as at 2 vol. per cent O_2. In pneumococci of the same seeding, collected after fifteen hours, the respiration rate was at 20 vol. per cent O_2 four and one-half times higher than at 2 vol. per cent O_2. The highest proportion found between the respiration at 2 and 20 vol. per cent O_2 was 1:5.4.

According to Warburg and Christian (1933) and Warburg (1934) oxygen transfer is not the physiological function of the yellow ferment, for the oxidation which it catalyzes is either quantitatively negligible or even damages the cells. Aerobic cells do not need the energy provided by the oxidation through the yellow ferment, though they also contain it in small amounts, since they obtain their life-sustaining energy by the haemin-ferment respiration. Anaerobic cells without haemin-respiration-ferment and containing large amounts of yellow ferment, obtain most of their energy by non-oxidative splitting processes and, because they lack catalase, they are easily damaged by the accumulation of the hydrogen peroxide which they form under aerobic conditions. (The damaging effect of an accumulated end-product of metabolism does not, however, indicate that this metabolism is not fundamentally necessary to cellular life.)

Since the respiration of pneumococci, catalyzed by the yellow ferment, was found to be dependent on oxygen tension, the ques-

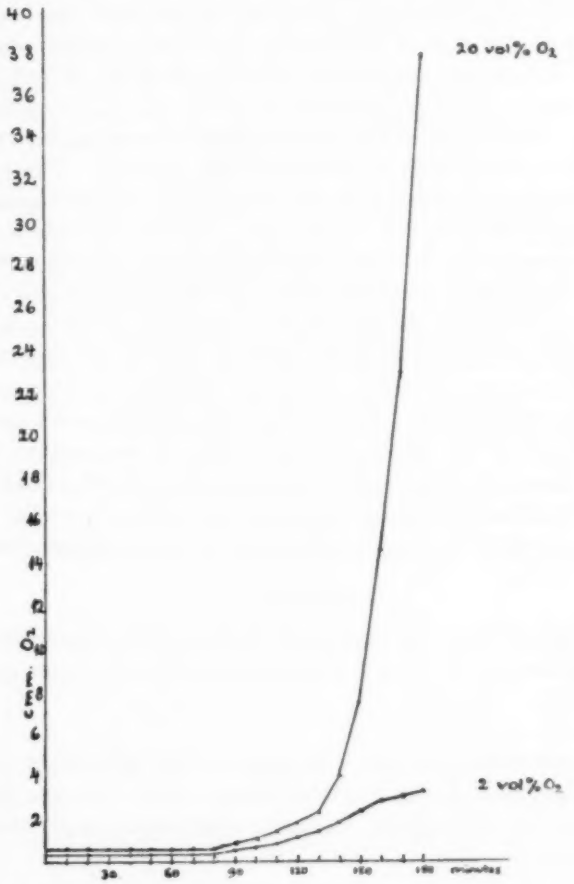

FIG. 3. GROWTH OF PNEUMOCOCCI (WEIGHT AT THE BEGINNING OF THE EXPERIMENT: 0.05 MG.) AT VARIOUS OXYGEN PRESSURES

The points in the curves show the experimentally determined amount of oxygen consumed in periods of 10 minutes.

tion arose: Is this oxidative process in the pneumococcus only an accessory chemical function of cells otherwise able to obtain their

energy by non-oxidative ferment reactions, for example, by the splitting of sugar, or is it a physiological, energy-supplying reaction, necessary for the building up of living substance?

To decide this question, we have determined the relation of growth to the rate of respiration at various oxygen tensions. Figure 3 shows the respiration of a growing broth culture of pneumococci at oxygen pressures of 2 vol. per cent and 20 vol. per cent. The points on the curve indicate the amount of oxygen consumed by the culture in periods of ten minutes. The growth of the bacteria is revealed by the increasing rate of respiration under otherwise identical conditions. As we see, the culture, which at a temperature of 37°C. is kept under a tension of 20 vol. per cent O_2, after an incubation time of one hundred and eighty minutes, consumes by respiration 38 cmm. oxygen in a ten-minute period while the same culture under a tension of only 2 vol. per cent O_2 respires after the same incubation time in a ten-minute period only 3.3 cmm. O_2. It is evident that pneumococci need the energy supplied by respiration and that, if respiration is inhibited, the energy obtained by fermentation is not sufficient to supply the amount of energy necessary for optimal growth. The velocity of growth is considerably lower at a low oxygen tension.

SUMMARY

Quantitative data are presented showing that respiration and growth processes of type I pneumococci depend upon oxygen tension.

The work here reported was done in the laboratory of Dr. Walter Kempner, Duke Medical School, and I am greatly indebted to him for many valuable suggestions and constant coöperation.

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THE RÔLE OF BACTERIA IN AUTOLYZING TISSUE[1]

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Autolysis by definition designates a process of self-digestion and specifically refers to the dissolution of tissues by enzymes present in the tissue cells, without the aid of micro-organisms. Wells (1925) has given an extensive review of the literature on this subject, the details of which are not within the scope of this paper.

Autolysis has been widely studied in animal fluids and tissues, the general thought being that such tissues when aseptically removed are sterile. Most investigators, however, who recognize the difficulty of maintaining asepsis in autolysis experiments, have added small amounts of toluene, chloroform, or other preservative to the autolyzing digest; the idea being that the preservative, while not absolutely inhibitory to enzyme action would nevertheless prevent the growth of bacteria. Bradley and his associates (1915) in an extensive review of autolysis covering many years, record no attempt to determine the possible presence of bacteria in their preparations. Much of their work was done with animal liver incubated with chloroform water or toluene water as a preservative. Recently Herron and McEllroy (1933) report that autolysis of beef liver in N/50 HCl to which a small amount of chloroform was added as a preservative, increased the potency of the liver material in the treatment of pernicious anemia. Mapson (1932) has also reported that the autolysis of beef liver at a pH of 4.5 to 5 increased the potency

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of the liver in its effects on the growth of the young rat. There is no record in these studies of attempts to determine the presence or absence of bacterial growth in the autolyzing preparations. It is apparently taken for granted by many investigators in this field that alterations in pH or the addition of small amounts of toluene or chloroform will prevent bacterial growth.

Wolbach and Saiki (1909) have shown that anaerobic bacteria are almost always present in aseptically-removed dog livers. They mention that the presence of these bacteria may account for many of the changes occurring in so-called autolysis of aseptically-removed liver. Mueller (1916) used sodium fluoride, chloroform, tricresol, and toluene successfully in the autolysis of blood but after attempts to autolyze liver in the presence of these antiseptics concluded: "It may be said that in some of the experiments in which liver was autolyzed there was apparently so much putrefaction, in spite of reasonable amounts of preservative, that positive results would have been questioned even if obtained." Gibbons and Reed (1930) taking elaborate precautions against contamination in the removal of tissues, studied the autolysis of fish muscle and guinea-pig kidney. They report finding large gram-positive bacilli in some of their digests in spite of the precautions taken.

In our studies on the bacterial flora of aseptically-removed pieces of dog liver and muscle (Trusler and Reeves (1934)) we have found that these tissues, particularly liver, almost invariably contain bacteria. Furthermore, among the bacteria commonly found are spore-forming, anaerobic, gas-forming bacilli, whose growth is not regularly prevented by alterations in pH or by the addition of weak antiseptic solutions.

We have repeated the work of Herron and McEllroy in order to determine if bacteria will grow under the conditions of autolysis which they specify. We have also conducted simple experiments to determine if variations in pH or addition of the antiseptics commonly used in studies in autolysis would inhibit the growth of bacteria in autolyzing preparations where the livers of slaughter house animals were used.

EXPERIMENTAL OBSERVATIONS

Herron and McEllroy (1933) report that when ground beef-liver is added to N/50 HCl in 1:5 proportion and allowed to incubate at 37.5 to 40°C. for ten days the potency of the liver in the treatment of pernicious anemia is increased 5 to 8 times. As a preservative, 25 cc. of chloroform per 1,000 grams of liver were added to the digest.

Experiment 1

A beef liver weighing 8.5 pounds was obtained from the abattoir and prepared as follows: 3 liters of scalding, autoclaved distilled water were poured over the surface of the liver. The liver was handled with aseptic surgical technique and ground in a sterile food chopper. 1,000 grams of the ground liver were placed in a sterile 10 liter flask. Five liters of N/50 HCl and 25 cc. of chloroform were added. The flask was thoroughly shaken and placed in the incubator at 37.5°C. to autolyze. The preparation was thoroughly shaken daily. Gross observations as to color, gas-formation, odor and appearance were made. Stained bacterial smears were made of the flask contents every other day. Determinations of pH were made of the digest at the beginning of the experiment and after ten days' incubation.

Results

Gross observations revealed that this concentration of HCl precipitated liver proteins and that the supernatant fluid was brown in color. The odor was never putrid or offensive. As incubation progressed it was noticed that the liver proteins became more finely divided and digested. After four days the fluid above the liver proteins cleared and in the unagitated state, appeared transparent and amber-colored.

The initial pH of the digest was in the neighborhood of 2. After ten days' incubation the pH of the flask contents was around 5.

There was an abundant growth of different kinds of bacteria in the digest. This is clearly demonstrated by photographs (plate 1) taken of smears of the flask contents stained by Gram's

method. Photograph 1 was taken after twenty-four hours' incubation. Photograph 2 was taken after ten days' incubation. Stained smears made of the flask contents after twenty-four hours' incubation revealed an occasional short-chained streptococcus and several large gram-positive bacilli.

Experiment 2

Since the autolyzed liver product available commercially is prepared from hog liver, it was of interest to see if hog liver autolyzed under the conditions specified by Herron and McEllroy would allow bacterial growth.

Pig livers were obtained fresh from the abattoir, prepared and autolyzed in the same manner described in experiment 1. In this case however, 2,000 grams of hog liver were autolyzed. Ten liters of N/50 HCl and 50 cc. of chloroform were added to the digest. Contrary to our expectations no bacteria were seen in stained smears made of the autolyzing preparation. This disparity in results might be explained in either of two ways.

It is possible that this particular autolyzing preparation at no time contained bacteria. It is likewise possible that bacteria were present in the digest at the time of its preparation, but were organisms of such low resistance that growth was prevented by the conditions of the experiment.

It was of interest to note that bacteria grew in the beef-liver digest in experiment 1 but failed to grow in the hog-liver digest in experiment 2. For a further study of these observations experiment 3 was carried out.

Experiment 3

For this experiment, 1 beef liver and 3 hog livers were obtained fresh from the abattoir and 3 different digests were prepared. One digest contained 2,000 grams of beef liver with 50 cc. of chloroform added as a preservative; one digest contained 2,000 grams of hog liver with 50 cc. of chloroform; and the other digest contained 1,000 grams of hog liver with 25 cc. of chloroform. The digests were prepared and incubated in the same manner as described above.

In this experiment no bacteria grew in the beef-liver digest, while large numbers of gram-positive bacilli grew in each of the hog-liver digests. Photograph 3 (plate 1) was taken of a stained smear made of the 2,000-gram digest of hog liver after four days' incubation. The bacterial flora of each of the hog-liver digests was similar morphologically.

The limited data in these experiments demonstrate no uniformity in results. In the first experiment bacteria grew abundantly in the beef-liver preparation. In the second experiment hog liver autolyzed in a similar way was free from bacterial growth. In the last experiment, however, the results were reversed; bacteria grew abundantly in each of the hog-liver digests while the preparation of beef liver was free from bacterial growth.

In the experiments of Mapson (1932) the liver was finely minced, brought to a pH of 4.5 to 5 and incubated at 37° to 40°C. for twenty-four hours. He states that under these conditions autolysis is facilitated and bacterial growth is inhibited. It seemed advisable to ascertain at what pH the growth of bacteria in digests of beef liver would be inhibited.

Experiment 4

Beef liver was prepared as described above. The ground liver was divided into 80-gram amounts and placed in 14 sterile Erlenmeyer flasks. Different solutions were made up with HCl and NaOH so that the final preparations each consisted of 80 grams of ground liver in approximately 250 cc. solutions with a pH range from 1 to 14 inclusive. The pH of the different digests was adjusted after the solutions were added to the liver so that the immediate buffering action of the liver proteins in the weaker solutions was accounted for. The flasks were thoroughly shaken and placed in the incubator at 37.5°C. to autolyze. The flasks were shaken daily and gross observations made on the lysis of the liver proteins, odor and presence of gas formation. Stained bacterial smears were made of the different digests throughout the period of incubation, namely, after 24, 48, 72, 216, and 264 hours respectively. This represented observations over a period of eleven days.

After eleven days' incubation the pH of each digest was again determined. Cultures were made using a good anaerobic culture medium (Trusler and Reeves (1934)) and the flasks with their contents were autoclaved. The preparations were then filtered and 2-cc. amounts of the filtrates were injected intravenously in a dog arranged so that kymographic tracings of blood pressure would indicate the presence of pressor amines. These amines are formed by the putrefactive action of bacteria upon animal tissue as reported by Barger and Walpole (1908–09).

An abundant bacterial growth occurred in all the preparations except the digest with an initial pH of 14. No bacteria were demonstrated in this digest at any time. Those preparations with an initial pH of 1 and 2 allowed only a limited growth of

TABLE 1

Showing the pH of the different digests at the beginning of experiment 4 and after eleven days' incubation

Initial pH.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
pH after 11 days' incubation.....	6.8	7.4	7.3	7.3	7.5	7.6	7.6	7.4	7.4	7.4	7.5	7.4	7.5	Still above 10

bacteria within the first twenty-four hours of incubation. After that the bacterial growth in all the digests was abundant. Morphologically there were many different kinds of bacteria present. The bacterial flora of the contents of the different flasks varied from day to day but the organisms predominating on all the smears were the gram-positive, spore-forming types. Photograph 4 (plate 1) was taken of a stained smear made of the digest with an initial pH of 1 after seventy-two hours' incubation.

Most of these preparations had a marked odor of putrefaction, but we wish to stress the fact that absence of putrid odor does not necessarily mean the absence of bacterial growth.

Cultures from all the preparations except the digest with an initial pH of 14 were positive for bacterial growth, demonstrated both grossly and by smear. The kinds of bacteria growing in

the cultures were similar to the flora growing in the original flasks.

Physiological tests of the different digests showed a marked pressor effect upon the blood pressure of the dog which was due in all probability to amines formed by the action of bacteria upon the liver protein.

TABLE 2

Showing the presence of bacteria in beef liver digests when various concentrations of antiseptics were used as preservatives (experiment 5)

Presence of bacteria was determined by smears stained by Gram's method. The results of cultures are discussed in experiment 5.

ANTISEPTIC	INCUBATION PERIOD IN HOURS				
	24	48	72	216	264
Chloroform water	+	+	+	+	+
5 per cent chloroform	+	+	+	+	+
25 per cent chloroform	+	+	+	+	+
Toluene water	+	+	+	+	+
5 per cent toluene	+	+	+	+	+
25 per cent toluene	+	+	+	−	−
5 per cent ethyl alcohol	+	+	+	+	+
25 per cent ethyl alcohol	+	+	+	−	−
50 per cent ethyl alcohol	+	−	−	−	−
0.5 per cent phenol	+	+	+	+	+
1 per cent phenol	+	+	+	+	+
5 per cent phenol	−	−	−	−	−

+ = vegetative forms present; and − = vegetative forms absent.

Experiment 5

Since it is apparently taken for granted by many investigators that the addition of toluene, chloroform and other antiseptics in small amounts will prevent bacterial growth, it was decided to test the truth of this conception so far as a single beef liver was concerned.

Twelve digests of beef liver were prepared in the same manner as described in experiment 4. Sterile water was combined with different antiseptics so that the final solution added to the 80 grams of liver equaled 250 cc. Table 2 presents the results of this experiment. It was found that bacteria grew in all the

digests where the concentration of the preservative was not sufficient to cause thorough coagulation of the liver protein. The flasks containing only chloroform or toluene water showed gas production and other gross evidence of bacterial growth. This was noted also in some of the stronger antiseptic concentrations. Usually there were no odors of putrefaction and several of the flasks showed no gross evidence of bacterial growth; nevertheless, smears made of these digests were positive for vegetative forms of bacteria. It is true that the stronger antiseptic solutions definitely inhibited bacterial growth. It can be seen by reference to table 2 that when beef liver is autolyzed in the presence of the stronger antiseptics there is at first a bacterial growth which, upon further incubation, disappears. For example where the digests contained 25 and 50 per cent alcohol and 25 per cent toluene as preservatives, bacterial growth was demonstrated for the first days of incubation, after which the digests contained no vegetative forms of bacteria. However, when the preparations were sub-cultured into routine anaerobic culture medium after eleven days' incubation, bacteria grew in all the cultures except those taken from the flasks to which 50 per cent alcohol and 5 per cent phenol had been added as preservative. Obviously, such strong antiseptics could have no place in studies of autolysis.

DISCUSSION

In these simple experiments we have shown that the addition of mild antiseptics does not regularly prevent bacterial growth in autolyzing preparations of fresh beef or hog livers as they are obtained from the slaughter house. We have likewise shown that a marked alteration in the initial pH of the digest does not regularly prevent bacterial growth.

We are not in position to make a definite statement as to the source of these bacteria. It is obvious that slaughter-house livers have surface contamination with many varieties of organisms. However, we did subject these livers to repeated washings with scalding water until the surfaces of the liver were coagulated. From this point the preparations were made with sterile

technique. Although these precautions cannot insure against surface contamination it is nevertheless true that two of the digests, one of hog liver and one of beef liver did remain sterile as shown by repeated smears and a culture taken after eleven days' incubation. It is possible that the absence of bacteria in these two digests might have been due to the preservative which was added. The preservative, however, did not prevent bacterial growth in numerous other digests prepared in a similar way.

We are of the opinion that the bacteria which grew in these autolyzing preparations were lying dormant in the livers of the living animals. We think it highly probable that they are present in some livers, absent in others. We have repeatedly demonstrated their presence in aseptically removed pieces of liver and muscle taken from the living dog. Wolbach and Saiki, Reith, Berg, Zau and Jobling and others have reported their presence in the tissues of the dog and other animals. We believe that these organisms are highly resistant strains of sporulating, gas-forming, anaerobic bacilli which are saprophytic in nature. They grow readily in dead tissue, (Trusler and Reeves (1934)).

We need not discuss further the source of the bacteria which grew in our autolyzing preparations. The fact remains that there were strains of bacteria present in the digests of slaughter-house livers which grew in the presence of preservatives unless the preservative was present in sufficient concentration to coagulate the liver proteins. Even in this latter instance bacteria grew to a limited extent. This phenomenon may be due to the fact that the bacteria grew in the center of masses of animal tissue where the preservative had not penetrated. Photograph 5 (plate 1) was taken of a stained smear made of a ground beef-liver digest which had been incubated twenty-four hours in the presence of 25 per cent toluene. After eight to ten days' incubation no vegetative forms of bacteria were demonstrated in smears made from this preparation. Nevertheless, subsequent culture of this digest was positive for bacterial growth.

It was observed in experiment 4 that, although the initial pH of the different digests ranged from 1 to 14, the pH determinations made of these preparations after eleven days' incubation

revealed that all but one of them was near the neutral point as shown in table 1. We have not as yet made sufficient study to explain this phenomenon. It seems logical however, that the change of reaction toward the neutral point may have been caused by the buffering action of degradation products resulting both from autolysis and bacterial digestion of the liver substance. It is possible that this tendency of the reaction to approach the neutral may explain the fact that spore-forming bacteria were able to grow in digests with such an initial wide range of pH.

The fact that bacterial contamination may influence the results of experiments on autolysis needs no comment. Herron and McEllroy (1933), however, have reported an interesting observation to the effect that autolysis of beef liver increases its potency in the treatment of pernicious anemia. We have shown that bacteria may grow in such liver digests without giving any gross evidence of putrefaction. It is interesting to note further that bacteria have been shown to possess an ability to synthesize vitamin B (Sunderlin and Werkman (1928)). Many reports have indicated that vitamin B may be closely related to, or associated with, the anti-anemic factor present in liver.

We wish again to emphasize the fact that any studies, involving the autolysis of animal tissue may be vitiated by growth of bacteria in the digests. Especially is this true if gross quantities of tissue emulsions are being studied. In none of our experiments did we use small quantities of finely-minced, widely-dispersed tissue suspensions. It is possible that under such conditions preservatives might be more effective. The problem is worthy of more careful investigation.

SUMMARY

1. Many past experiments on the subject of tissue autolysis have not given proper consideration to the possible presence of bacteria in the digests.

2. Autolyzing digests of hog and beef liver obtained fresh from the slaughter house, have been shown to contain highly resistant strains of spore-forming bacteria.

3. Alterations in pH and the use of antiseptics as preservatives

have failed to prevent the growth of bacteria in most of the autolyzing preparations we have studied.

4. In our experiments, sterility of the digests could be determined only by bacterial smears and cultures. We repeatedly found anaerobic spore-forming bacilli growing in digests which were free from putrid odors and showed no gross evidence of bacterial growth.

The authors wish to express their appreciation for the kindly assistance and criticisms given by Dr. Harold M. Trusler, Director of Research.

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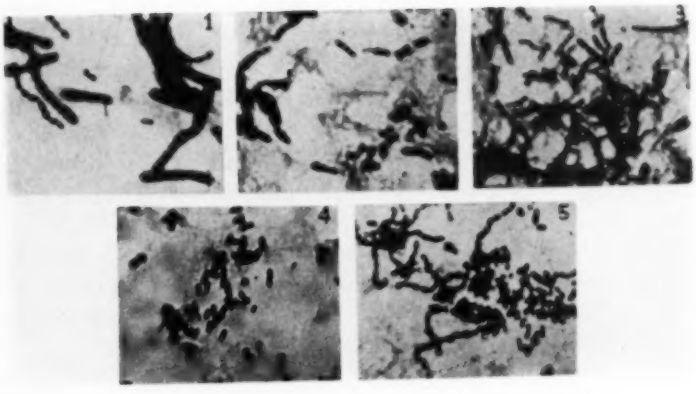
PLATE 1

PHOTOMICROGRAPHS OF BACTERIAL SMEARS MADE OF DIFFERENT DIGESTS OF LIVER AUTOLYZED UNDER VARIOUS CONDITIONS. THE SMEARS WERE STAINED BY GRAM'S METHOD

1. Beef liver autolyzed according to the specifications of Herron and McEllroy after twenty-four hours' incubation.
2. The same preparation as 1 after ten days' incubation.
3. Hog liver autolyzed according to the specifications of Herron and McEllroy after seventy-two hours' incubation.
4. Beef liver autolyzed at an initial pH of 1 after twenty-four hours' incubation.
5. Beef liver autolyzed with toluene as a preservative after twenty-four hours' incubation. The amount of toluene equaled 25 per cent of the digest.



(James R. Reeves and Hugh E. Martin: Bacteria in Autolyzing Tissue)

STUDIES ON BACTERIAL PIGMENTATION[1]

I. HISTORICAL CONSIDERATIONS

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In 332 B. C., during the siege of Tyre by Alexander and his army of Macedonians, the soldiery became greatly concerned by the appearance of blood-like droplets on bread as it was broken. So great was the alarm that the soldiers and king alike were about to give up the siege since they considered this phenomena as a portent of their destruction. Upon having this brought to his attention, Aristander, one of the king's most skillfull soothsayers, interpreted it to mean that since the droplets of blood were on the inside of the loaf, it indicated destruction for those within the walls. If the blood had been on the outside of the loaf, it would have portended evil to the army outside of the walls. Taking courage from these interpretations, the Macedonians continued their siege and the city of Tyre fell (1).

To the modern scientist and bacteriologist, this "miracle" can have but one clear and logical explanation. The growth of what is now known as *Serratia marcescens*, the red pigmented bacterium which grows profusely on bread and even today causes what is commonly called "bloody bread." Later events give proof to this theory.

Of even greater antiquity, although any evidence to prove the fungus nature of the "blood" is lacking, is the phenomena which gave rise to the superstition of the "sacred nature" of beans. Because of this belief they were barred from the diet of many of

[1] A portion of a thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The Pennsylvania State College. 1935.

the early races. According to Lucian (2) "if beans are placed in the moonlight for a certain number of nights you will make blood." Among the Athenians, beans were used for balloting for the high officers.

Several instances of "blood" appearing on broken bread are recorded before any great consideration was given it. For instance, in 583 A. D. (3) "blood" was seen "to flow" from broken bread in Tours, France; in 1091 (3) in Zweifalten, Germany, "blood" was noticed "on" bread and in 1163 similar phenomena were reported from LaRochelle. However, while during a mass in Alsen Denmark in 1169 (3) a priest noticed "blood" on the Host. He became alarmed and showed it to the chief priest who predicted that serious trouble lay ahead for the church, and that blood of Christians would be shed. True to his prophecy, within a fortnight an invading army of pagans overthrew the state, destroyed the churches and placed all the Christians in slavery.

This is only the first of many similar occurrences which were to follow that gave rise to at least one important ecclesiastical custom. It is easy to understand how this pigmented organism which is found in air, soil and water and grows abundantly on all farinaceous foods found excellent conditions for development on the sacrament wafers left in the damp and unhygienic churches, and other places of the middle ages.

In 1199 (4) at Augsburg a woman kept a wafer of communion in her mouth. Upon arriving home she covered it with wax and kept it. When it was opened later it was found that the wafer had become "flesh and blood." It was thus guarded as a miracle at the cloister of the Holy Cross.

In 1200 (4) Alexander of Halle relates that "blood flowed" from the Host as it was broken at holy communion and filled a dish. The pieces of the Host were laid together, whereupon they closed into one mass and the "blood" was reabsorbed. In 1201 (5) a herdsman at Steffenshagen took a wafer that he had secured at Doberian church to his home and placed it in the staff with which he guarded his herd. It was discovered afterwards that the wafer had become "bloody"; it was taken to the Doberian church which soon became a place for pilgrimage and an octagon-shaped chapel was built for the Holy Blood.

Castle Chum, in the kingdom of Valencia, was besieged by the Sacracens in 1239 (4). So that they might fight in a calm and collected manner, six of the inhabitants wished to have communion which was being administered to them when a sudden attack interrupted the "feast." The priest wrapped the wafers in a cloth and after the attack, which was repulsed, the men returned to finish the communion to find the wafers "spotted with blood." The wafers were placed on a donkey which had been obtained from the invaders' army. It was given free rein and accompanied by the priest with candles. It went to Daroca, the birth place of the priest who had consecrated the wafers. Upon arrival there the donkey fell dead. The Host remained fresh for a long time.

A church in Bielitz became famous because of a "miracle" which occurred there in 1247 (6). A woman took a consecrated wafer from the church in her mouth, and later sold it to Jews who stabbed it. When "blood" appeared on it the Jews returned it to the girl who admitted the felony.

In 1249 (6) a barmaid in Zehdink placed a consecrated wafer under a beer cask. Subsequently drops of "blood" were found under the cask where the wafer had been placed. Ehrenberg accredits this coloration to the presence of a fungus *Palmella sanguinea*.

The miracle of Bolsena (7) occurred in 1264 as follows: A priest who had not entirely accepted the truth of the "transubstantiation" was blessing the elements of the Host and drops of "blood" fell from it upon his robes. Upon his attempt to conceal the spots he made larger blots which took on the appearance of the Host. The robe was exhibited for some time in Civita Vecchia. Becoming convinced of the "transubstantiation" by this happening, Pope Urban IV instituted by Papal Bull the feast of the Corpus Christi for the whole church, to be held on Thursday after Trinity Sunday.

In 1290 (4) at Easter, a woman took a consecrated wafer from the church and sold it to Jews who boiled it without injury then stabbed it. The wafer became red in color and was observed by Christians who burned the Jew to death.

Similar occurrences took place in the various cities of Europe

during the century following. The result was that many unbelievers lost their lives, and some of the executions took place on a wholesale scale. Scheurlen (8) stated in this connection, "dieser Saprophyt mehr menschen ungebracht hat als mancher pathogene Bacillus."

Aside from the appearance of "blood" on the sacrament wafers there are many references to its appearance on bread in other places, as in Sienna in Poland, where "blood" was found on bread and when cut with a knife drops of it fell on the table. This occurred in 1550 and in 1555 at Newendorf "blood" flowed from bread and cakes.

The most important epidemic of spotted bread from our point of view, since it led to scientific explanation of the phenomena in the face of superstitions and supernatural causes accepted at that time, was that in Padua in 1819. The infection became epidemic and aroused much terror and speculation. The outbreak first appeared at the home of a peasant on August 2, 1819, with red spots on corn porridge. This was discarded but the next day spots appeared on fresh batches. A priest was called in to bless the place but in spite of this the epidemic continued and in fact, became worse. Due to good publicity the phenomenon received widespread attention and much discussion. The illiterate considered it an omen of the Almighty of punishment to come for speculation on the grain market causing an increase in prices. So general became the excitement that a commission was appointed to determine the cause of the trouble. Professors of the University, health officers and police officials coöperated under the direction of Doctor Vincenzo Sette, a medical officer. A member of the commission, Bartelomeo Bizio, promptly recognized the fungus nature of the coloring matter and proceeded to infect other farinaceous materials with the organism isolated. These showed bloody spots within twenty hours. He reported his findings in the "Gazetta Privilegiata di Venezia" on August 24, 1819 (9). In 1823 he published the results of his further studies on the organism and a description of it. He named it *Serratia marcescens*, the generic name, a tribute to Serrafino Serratia, the first to run a steamboat on the Arno, and the species

name indicating decaying or putrefying. In 1827 he reported a summary of his findings in regard to the chemical nature of the pigment.

Further investigations by the commission, including a chemical investigation of the colored substance, were reported by Dr. Sette in his Memoirs in 1824 (10). He named the causative organism *Zoaglactina imetropha* which in Greek means "living slime on food."

In the years that followed others investigated what may be presumed to be the same organism. In 1848 Ehrenberg (11) reported on an outbreak of "bloody bread" which occurred in Berlin. He made extensive studies of the organism but changed the name to *Monas prodigiosa*, because he did not agree with Sette's nomenclature and apparently was not acquainted with Bizio's paper since he does not refer to it.

Other names were given the red organism by Montagne (1852) (12), *Palmella prodigiosus*; F. Cohn (1872) (13), *Micrococcus prodigiosus*; Schroeter (1872) (14), *Bacteridium prodigiosus*; Trevisan (1887) (5) *Bacillus imetrophus*; De Toni and Trevisan (1889) (16), *Bacillus marcescens*; to cite a few.

In 1918 Buchanan (17) returns credit for naming the organism to its rightful owner, Bizio, and today according to the classification of the Society of American Bacteriologists the organism is called, *Serratia marcescens*. The first appearance of this name in Bergey's Manual of Determinative Bacteriology is in the 1923 edition.

The colorful history of this organism has been briefly traced and its influence upon history indicated. Many other saprophytic organisms doubtless have an equally interesting genealogy were we to consider them. There are those who will probably take issue with the liberal interpretation of some of the accounts reviewed herein, but from a purely scientific point of view there can be little doubt as to the logic of concluding that this minute and harmless saprophyte has been responsible for many sleepless nights, needless murders and executions and important pages in history.

ACKNOWLEDGMENT

The author gratefully acknowledges the suggestions obtained from a paper by Harrison (18) "The Miraculous Micro-organism."

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VARIATIONS IN THE ELECTROPHORETIC MOBILITIES OF ESCHERICHIA, AEROBACTER AND "INTERMEDIATE" STRAINS

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Since, by cultural methods, it is often difficult to obtain any well defined differentiation between the *Escherichia, Aerobacter* and "intermediate" organism it was thought that a determination of the zeta potential of the various strains comprising these groups might be of assistance in their identification. Chapman (1929) has shown the zeta potential of various strains of *Escherichia coli* to be constant. Numerous experiments conducted by us show that this is true, not only for *Escherichia coli* strains, but for *Aerobacter aerogenes* as well.

One hundred and twenty-seven strains of *Escherichia, Aerobacter* and "intermediates" were isolated from oysters and oyster water,[1] water, urine, feces, and necropsies, or obtained from the Tittsler collection. They were distributed according to Bergey's Determinative Bacteriology (1934) and Tittsler and Sandholzer's (1935) review groups as shown in table 2.

METHOD

A number of these cultures have been used by us in previous investigations. The cultures were grown on proteose-peptone agar slants of an approximate pH of 6.8 for twenty-four hours. The growth was washed off the surface with distilled water, pH 6.8, homogenized by gentle shaking, filtered through cotton and centrifugalized at about 2700 r.p.m. for 45 minutes. The super-

[1] The writer wishes to express his appreciation to T. F. Dozois for his isolation of these cultures.

natant fluid was removed and the cells resuspended and rewashed twice.

Only distilled water of pH 6.8 was used in the work. A pH determination (W. A. Taylor & Company Slide Comparator) was made on each suspension directly after the last washing and before an electrophoretic reading was made. On 65 suspensions the pH was also determined after the readings were obtained. In all instances the pH of the suspension remained at 6.8. When it was necessary to store the suspensions for any length of time they were held at 5°C. since Dozois and Hachtel (1935) have shown that storage at higher temperatures may affect the zeta potential.

Zeta potential measurements were made with the Kunitz modification of the Northrop-Kunitz microcataphoresis cell (1928), with a microlamp, a Bausch and Lomb 8 mm., 0.50 n.a. 21X objective and a 10X eyepiece. Twenty readings were made at two stationary levels, i.e., 0.21 and 0.79 of the inside depth of the cell. An applied potential of 118 volts, which gave a drop of 2.5 volts through the cell, was used. This, with a series resistance of 1100.84 ohms, would duplicate in effect an applied potential of 125 volts supplied by Radio B batteries. The electrophoretic mobilities are given in seconds per 0.5 mm. Each reading recorded is the average obtained from 3 or more separate studies carried on at different times, conditions being duplicated as nearly as possible each time. On any strain in which the readings varied more than 4 per cent the work was repeated. In some instances as many as 9 separate determinations were made.

RESULTS

Although table 1 is only a partial record of the total results obtained it is sufficient to show that the zeta potential of the various strains of *Escherichia*, *Aerobacter* and "intermediates" is a characteristic constant for each strain. By careful control of the period of growth, the pH of the growth medium and suspension solution, and by using the same technique in the operation of the apparatus for determination of the electrophoretic

TABLE 1

Tabulation of the electrophoretic velocities of strains of Escherichia, Aerobacter, and "intermediates"

GENUS AND SPECIES	STRAIN	ELECTROPHORETIC VELOCITY			
		seconds per 0.5 mm.	*seconds per 0.5 mm.*	*seconds per 0.5 mm.*	*seconds per 0.5 mm.*
Escherichia coli	1	10.6	10.4	10.6	10.5
	2	13.0	12.8	12.8	12.9
	3	14.8	14.6	15.0	14.9
	4	13.1	13.5	13.0	13.3
	5	12.2	12.5	12.0	12.3
Escherichia communior	1	13.5	13.6	13.2	13.4
	2	11.9	11.9	12.3	12.2
	3	12.9	13.1	13.3	12.8
	4	13.4	13.0	13.0	12.8
	5	13.7	14.1	14.0	13.9
Escherichia neapolitana	1	14.1	13.8	14.1	13.7
	2	13.5	13.3	13.5	13.2
	3	12.3	12.5	12.7	12.4
Aerobacter aerogenes	1	14.5	14.7	14.2	14.6
	2	15.4	14.9	14.3	14.8
	3	11.7	11.7	11.4	11.6
	4	12.8	13.1	13.1	12.6
	5	13.3	13.5	12.9	13.1
Aerobacter liquefaciens	1	12.4	12.4	12.0	11.9
	2	17.2	16.8	16.7	16.9
	3	15.6	15.3	15.3	
	4	14.8	14.7	15.1	15.1
Aerobacter oxytocum	1	11.5	11.8	11.5	11.6
	2	11.4	11.5	11.4	11.6
	3	13.5	13.5	13.6	13.3
	4	14.7	14.4	14.6	14.3
"Intermediates"	1	13.9	14.3	14.1	14.3
	2	11.5	11.0	11.3	11.6
	3	15.3	15.7	15.5	15.5
	4	16.7	16.4	16.4	16.5
	5	14.8	15.3	15.1	15.1
	6	15.3	15.6	15.4	15.4
	7	12.3	12.3	12.3	12.5
	8	13.4	13.6	13.8	13.5
	9	13.9	14.4	14.0	13.8

migration velocity, it was found that the zeta potential of the various strains remains constant over a long series of transplants. Whether the strain was freshly isolated or carried for a period of time on an artificial growth medium apparently had little influence on the results. Although the electrophoretic migration velocity of a single strain remains constant, there is, nevertheless,

TABLE 2

Tabulation of the average, minimum, and maximum electrophoretic velocities of species of Escherichia, Aerobacter and "intermediates"

CULTURE	TOTAL NUMBER	ELECTROPHORETIC VELOCITIES		
		Average velocity	Minimum velocity	Maximum velocity
		seconds per 0.5 mm.	*seconds per 0.5 mm.*	*seconds per 0.5 mm.*
Escherichia	47	13.2	18.9	9.2
coli	10	12.2	15.0	10.6
enterica	9	14.5	18.9	12.3
anaerogenes	6	13.2	16.7	11.0
communior	9	13.6	14.1	11.9
neapolitana	7	13.2	14.1	12.3
communis	8	13.2	18.5	11.9
alba	8	12.3	14.1	9.2
Aerobacter	49	12.3	17.2	11.0
aerogenes	14	12.3	15.4	11.4
oxytocum	10	13.2	14.7	11.4
cloacae	8	12.2	14.7	11.0
liquefaciens	12	13.7	17.2	11.9
levans	5	12.7	13.2	11.4
"Intermediates"	32	13.2	16.7	11.0

a wide variation in velocity between the strains of the same species. Because of this variation, and since the zeta potential of one strain of one species may be very near to, or often identical with, to the potential of a strain of another species it is impossible to identify a strain or a species by its electrophoretic migration velocity.

Although the number of species studied is not large, they repre-

sent many of the organisms most commonly isolated from the sources indicated. Had more extensive studies been made, it is hardly to be expected, in view of our observations, that the results would have varied greatly from the recorded determinations. As shown in table 2, the average electrophoretic migration velocity of the 7 species of *Escherichia* was found to be 13.2 seconds, the minimum 18.9 seconds and the maximum 9.2 seconds. The average migration velocity of the 5 species of *Aerobacter* was 12.3 seconds, minimum 17.2 seconds, maximum 11.0 seconds; and the average velocity of the 32 strains of the "intermediates" was 13.2 seconds, minimum 16.7 seconds and maximum 11.0 seconds. It is interesting to note that although the range between the minimum and maximum velocity was greater for *Escherichia* than for the "intermediates" the average migration velocity of the two is the same, which is less than the average found for *Aerobacter*. All attempts to correlate electrophoretic migration velocities with specific cultural reactions proved unsuccessful.

SUMMARY

The electrophoretic migration velocity of the individual strains of *Escherichia*, *Aerobacter* and "intermediates" is constant, but there is a wide range in velocity between strains of the same species. Since a strain of one species may have the same migration velocity as a strain of another species; and, since the average velocity of one genus may be very near to that of another genus, it has not been possible to differentiate between *Escherichia*, *Aerobacter* and the "intermediates" by a study of their electrophoretic migration velocity.

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A STUDY OF THE SPECIES LACTOBACILLUS PLANTARUM (ORLA-JENSEN) BERGEY ET AL.[1]

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In systematic work it is essential that the characters used to define species shall be of such a nature that at least the majority of the strains can be properly and readily identified. In only relatively few cases have descriptions of species of bacteria been sufficiently comprehensive to include even known variations and, as a result, new strains or variants of species are frequently described as new species. For such reasons it was found difficult to arrange a key for the species of the genus *Lactobacillus* for Bergey's *Manual of Determinative Bacteriology* (1934). Many so-called species could not be separated from one another. This situation was particularly evident in those species of non-gas-producing organisms normally found in plant materials or in soil.

In a study of over four hundred strains of non-gas-producing organisms isolated from fermenting substances no characters were observed which could satisfactorily be used to separate the cultures into groups that corresponded to described species. It seemed, therefore, that nearly all of the descriptions that have been given are not only too incomplete, but are also too restricted in nature and that more inclusive descriptions should be used. The description of the species *Streptobacterium plantarum* Orla-Jensen (1919) syn. *Lactobacillus plantarum* Bergey *et al.*, is the

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first one given in this group that is sufficiently general to be satisfactory.

The name was applied by Orla-Jensen to a series of strains of bacteria isolated from butter, milk, cheese, fermenting potatoes, beets, cabbage and dough. Complete fermentation characters on forty-four strains were presented. Fermentation usually varied somewhat with the source of nitrogen. Inactive lactic acid was produced by the majority of strains, although a few produced an excess of dextro-lactic acid. Studies on changes produced in nitrogenous foods show that the strains do not split the proteins to any degree. More or less complete descriptions of other characters were given. The species differs but slightly from *Streptobacterium casei* Orla-Jensen which is described, in contrast to *Streptobacterium plantarum*, as producing dextro-lactic acid and as usually preferring lactose to maltose and sucrose.

Variations in the sugar fermentations are the only characters of a sufficiently constant nature to raise the question of establishing more than one species in the group of cultures included under the name *Streptobacterium plantarum*. When the results secured by Orla-Jensen are summarized, it is found that if the group is split, it should be on the basis of the fermentation of the sugars xylose, arabinose, or raffinose. If these sugars are selected for use in separating the strains into species, there would be as many species as there are possible combinations of characters. Strains that produce a vigorous xylose fermentation can be separated into 67 per cent weak and 33 per cent strong arabinose fermenters, or into 17 per cent weak and 83 per cent strong raffinose fermenters. Strains of vigorous arabinose fermenters can be separated into 74 per cent weak and 26 per cent strong xylose fermenters, or into 24 per cent weak and 76 per cent strong raffinose fermenters. Variations are noted in the fermentation of these sugars.

Over four hundred strains of non-gas-producing, short-rod forms from various fermenting substances have been used in our study. These studies have included morphology under different growth conditions, growth characters, resistance to heat and to

salt, hydrogen ion relationships, temperature relationships, products of fermentation, and ability to ferment carbon compounds. Morphology varied with conditions of growth and under like conditions the majority of strains were the same. Although other differences were noted, the greatest variation in characters was found to occur in ability to ferment sugars. Every gradation from a definite failure to ferment to a definite

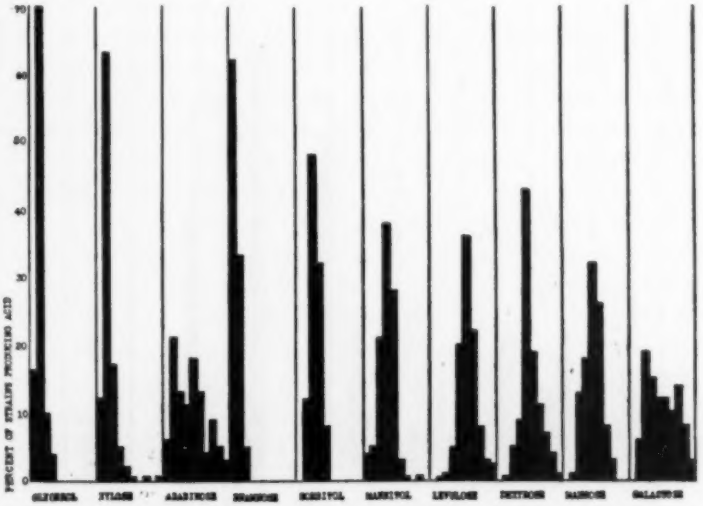

FIG. 1A. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY STRAINS OF LACTOBACILLUS PLANTARUM EXPRESSED AS PER CENT OF STRAINS PRODUCING FROM 0 TO 9 CC. OF ACID IN 10 CC. OF MEDIUM

Ten spaces used for each sugar signify in the first block per cent of strains producing no acid, the second, per cent of strains producing 0.1 to 1 cc. of acid, the third space 1.1 cc. to 2.0, etc. The tenth space is used for percentage of strains producing 8.1 to 12.5 cc.

positive fermentation was noted for nearly all of the carbon compounds (fig. 1, *a* and *b*).

Almost all strains fermented glucose, levulose, mannose, galactose, sucrose, maltose, lactose, raffinose and salicin, and to a lesser extent, sorbitol, mannitol, dextrin and glycerol. The majority of strains did not ferment rhamnose, starch and inulin. A few strains had little or no action on arabinose, while a greater

number had little or no action on xylose. Melezitose was about the only carbohydrate in which an approach to a clear-cut positive or negative fermentation was obtained. The same range of variation occurred even where cultures were obtained from identical materials.

If this group of cultures is divided into more than one species on the basis of fermentation characters, the carbon compounds

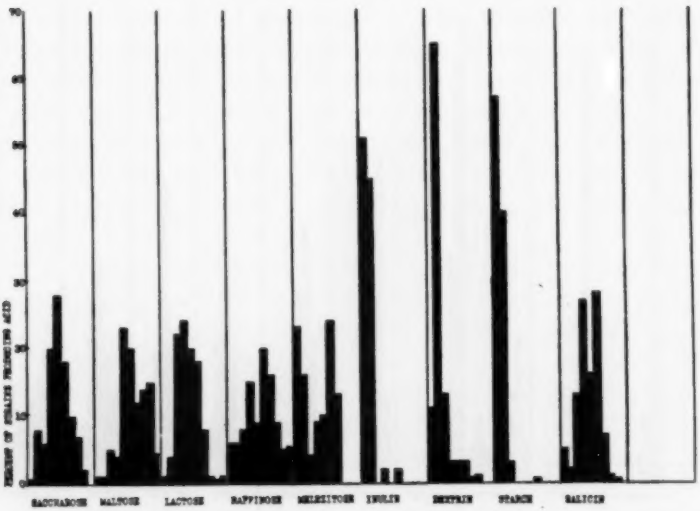

FIG. 1B. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY STRAINS OF LACTOBACILLUS PLANTARUM EXPRESSED AS PER CENT OF STRAINS PRODUCING FROM 0 TO 9 CC. OF ACID IN 10 CC. OF MEDIUM

Ten spaces used for each sugar signify in the first block per cent of strains producing no acid, the second, per cent of strains producing 0.1 to 1 cc. of acid, the third space 1.1 cc. to 2.0, etc. The tenth space is used for percentage of strains producing 8.1 to 12.5 cc.

which could be used would be melezitose and arabinose and possibly xylose and galactose; but when characters are studied carefully, little justification can be found for dividing the group. Even the fermentation of melezitose merely separates the less active strains from the more active strains. Since this sugar has not been used by previous workers and the separation has little importance, it seems more reasonable to consider this as a strain variation.

Among the strains studied, cultures have been found which agree in their characteristics with many of the closely related so-called species described. The name *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* appears to be the most logical selection of a name for this species, even though several strains had previously been described and named. However, the name mentioned is the first one given to a well described and inclusive species. Described species which appear to be wholly or in part identical are as follows:

Bacillus pabuli-acidi II Weiss[3] is poorly described, and the name given is in the form of a trinomial. The species was considered to be related to *Bacillus* α of von Freudenreich now called *Lactobacillus casei* (Orla-Jensen) Holland. Possibly, strains described by Weiss were related to *Lactobacillus casei* rather than to *Lactobacillus plantarum*.

Six of the so-called species described by Henneberg in 1903 appear to belong to *Lactobacillus plantarum*. The descriptions by Henneberg are very complete in certain respects, but incomplete in others. Descriptions differ but slightly, and variations may possibly be dependent upon growth conditions. Henneberg's species differ from one another primarily in respect to their fermentation of carbon substances. The first of these, to which he has applied a trinomial *Bacillus cucumeris fermentati* was later renamed *Lactobacillus cucumeris* by Bergey *et al.* in 1923. It is the only well known name and the only one of the group of which representative cultures are available. It is described as fermenting arabinose and dextrin vigorously. Two cultures received from Henneberg show no significant differences from cultures of *Lactobacillus plantarum*. Since the binomial *Lactobacillus cucumeris* was proposed later than the binomial *Streptobacterium plantarum* Orla-Jensen 1919 (now *Lactobacillus plantarum*), the latter name is the valid one. Descriptions of the other species are similar. *Bacillus Wortmanni* is reported as fermenting alpha methyl glucoside. *Bacillus Listeri* shows a weak fermentation of arabinose. *Bacillus Maerckeri* shows a

[3] Bibliographic references to all of these names will be found in Bergey (1934), pages 307–312.

weak action on arabinose and alpha methyl glucoside but a strong fermentation of dextrin. *Bacillus Leichmanni II* is an inulin fermenter, a weak arabinose fermenter but a high acid producer. Since it is described as granular, there may be some doubt whether or not this should be regarded as identical with the species *Lactobacillus plantarum*. *Bacillus Leichmanni I* and *III* either fail or slightly ferment arabinose, lactose and raffinose, and do not curdle milk. They seem to be more closely related to the species *Lactobacillus Delbrueckii* described by Leichmann as *Bacillus Delbrücki* than to *Lactobacillus plantarum*. Possibly they are intermediate between *Lactobacillus plantarum* and *L. Delbrueckii*. Unfortunately the type of lactic acid formed was not determined for any of these types. *Bacillus Beijerincki* is a weak fermenter of arabinose and trehalose and has no action on lactose, mannitol and milk. It is a weak strain more like *B. Leichmanni I* and *III* and *B. Delbrücki* in its sugar fermentations.

From this brief summary, it may be noted that species differentations have been based primarily upon differences in sugar fermentations, although in many cases these are not definitely positive or negative.

Two other species, *Lactobacillus conglomeratus* Beijerinck (1901) (1908) and *Lactobacillus densus* Beijerinck appear to be identical with *Lactobacillus plantarum*, but the descriptions given are very incomplete.

Three other names have been given to organisms that apparently belong to this species subsequent to Orla-Jensen's description of *Lactobacillus plantarum*, namely *Lactobacillus pentosus* and *Lactobacillus arabinosus* as described by Fred, Peterson and Anderson and *Bacterium busae-asiaticae* as described by Tschekan. The latter is apparently identical with *Lactobacillus plantarum*. *Lactobacillus pentosus* and *Lactobacillus arabinosus* were described shortly after the publication of Orla-Jensen's work and are more properly regarded as varieties of this species, the differentiation being based on the fermentation of xylose. In an unpublished paper by Fred and Peterson (1935) complete biochemical characterization is given of non-gas producing rods from fermenting vegetables. In this manuscript, they have referred to the cul-

tures by the names *Lactobacillus plantarum* and *Lactobacillus cucumeris.*

CONCLUSION

It appears logical to retain the name *Lactobacillus plantarum* as the species name for bacteria having the following characteristics:

Lactobacillus plantarum (Orla-Jensen) Bergey *et al.*

Synonyms in whole or in part: *Bacillus pabuli-acidi II* Weiss, *Bacillus cucumeris fermentati* Henneberg, *Bacillus Wortmanni* Henneberg, *Bacillus Listeri* Henneberg, *Bacillus Maerckeri* Henneberg, *Bacillus Leichmanni II* Henneberg, *Bacillus Beijerincki* Henneberg, *Lactobacillus pentosus* Fred, Peterson and Anderson, *Lactobacillus arabinosus,* Fred, Peterson and Anderson and *Bacterium busae-asiaticae* Tschekan and probably *Lactobacillus densus* Beijerinck and *Lactobacillus conglomeratus* Beijerinck.

Morphology: Non-motile, Gram-positive rods, ordinarily 0.7 to 1.0 by 3.0 to 8.0 microns, occurring singly or in short chains with rounded ends. Under favorable growth conditions these organisms tend to be short rods. Under adverse conditions they tend to be longer; for example, in tomato juice agar at 45°C. (Pederson, 1929). In fermenting vegetables, the organisms tend to become longer as the acidity becomes greater. The organisms are usually longer in milk than in broths. Differences in morphology are well illustrated by Orla-Jensen.[4]

Gelatin colonies: No growth.

Gelatin-yeast extract-glucose stab: Filiform growth. No liquefaction.

Agar slant: Growth, if any, is very faint.

Broth: Turbid, clearing after a few days. A few strains flocculate.

Litmus milk: Acid, usually coagulated.

Potato: No growth.

Indol not formed.

Nitrates not reduced.

The majority of strains form acid in glucose, levulose, mannose,

[4] See plates 41 and 45, Orla Jensen, 1919.

galactose, arabinose, sucrose, maltose, lactose, raffinose, and salicin, and to a lesser extent, in sorbitol, mannitol, dextrin, glycerol, and xylose. Rhamnose, starch and inulin are usually not fermented.

Lactic acid, usually inactive, with only small quantities of acetic acid and carbon dioxide, are formed in the fermentation of hexose sugars. Acetic and lactic acid are produced from the pentoses. Forms up to 1.2 per cent acid in broth.

Salt tolerance: Usually grow in salt up to 5.5 per cent.

Microaerophilic.

Optimum temperature 30–35°C. Maximum 40°C. Minimum 10°C.

Thermal death temperature: 65° to 75°C. for 15 minutes.

Sources from which isolated: milk, cheese, butter, kefir, feces, fermenting potatoes, beets, corn, chard, bread dough, sauerkraut, cucumber pickles, tomato pickles, cauliflower pickles and spoiled tomato products.

Habitat: Widely distributed in nature, particularly in fermenting plant and animal products.

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ON THE DETECTION OF NITRATE REDUCTION[1]

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For 30 or 40 years one of the features regarded as most important in the characterization of bacteria has been ability to reduce nitrates. This is so true that in nearly any paper in which some species is described, a statement is given as to whether or not it reduces nitrate and in most manuals of determinative bacteriology this characteristic is given under the descriptions of nearly all species.

The majority of such books and scientific papers, however, merely make a categorical statement "nitrates reduced" or "nitrates not reduced," as if it were so simple a determination as not to require specification of the methods. Unfortunately, this is not the case. It has been pointed out frequently that there is no simple procedure by which the ability of an organism to reduce nitrate can be categorically determined. There are, to be sure, simple tests which show, in the case of a positive reaction, that nitrate reduction has occurred; but to show that reduction does *not* occur is a complicated undertaking which is rarely attempted in a routine way.

As a matter of fact, in a medium containing nitrate, there are many possible ways in which action on this substance may occur, to wit:

(1) Reduction to nitrite, ammonia, and free nitrogen, with or without accumulation of the ammonia and nitrite; (2) reduction to nitrite, then to ammonia as a final end product, with or without accumulation of the nitrite; (3) reduction to nitrite, followed by assimilation of the nitrite, with or without its accumulation in

[1] Paper approved May 8, 1935 as Journal Paper No. 91 by the Director of the New York Agricultural Experiment Station.

detectable quantities; (4) reduction to ammonia, followed by assimilation of the latter, with or without accumulation of either nitrite or ammonia; (5) assimilation of the nitrate directly without its previous reduction; (6) no action on the nitrate. A few other alternatives may be possible (such as reduction to ammonia or free nitrogen without intermediate stage) although they have never been demonstrated and do not seem likely on theoretical grounds. Further variations occur, because in any of the above instances except No. 6, the nitrate may be either completely or only partially utilized. It has, in fact, never been proved that other nitrogenous compounds, such as amino acids, hydroxylamin or hyponitrous salts, may not be formed from the nitrate. With all these variations possible, it is easy to see that it is no simple matter to learn just what happens in a nitrate medium.

Inquiry of teachers in bacteriology indicates that when they are having their students identify organisms by means of some guide in determinative bacteriology, they have them regard an organism as reducing nitrate if nitrite or visible gas is produced in some nitrate medium; otherwise, as not causing reduction. Now, it may be entirely true that the production of nitrite or gas in a nitrate medium is in many instances a feature of decided diagnostic importance. Quite possibly this is all that is intended by the statement "nitrates reduced" or "nitrates not reduced" when it occurs in the description of a bacterial species. If this is the actual meaning, however, the statement has been very loosely worded and should not be left unexplained in any manual frequently consulted by students of bacteriology. As a matter of fact, negative results in such a determination are open to many sources of error if they are to be interpreted as non-reduction of nitrate.

SOURCES OF ERROR

Gas production

In the interpretation of gas production in a nitrate medium, one must first of all remember that even a positive reaction may be misleading, provided there is any ingredient in the medium other than nitrate from which gas could be produced. It would

obviously be out of the question to study the colon organism, for instance, as to its ability to produce gas from nitrate by growing it upon any medium also containing sugar. When this contingency is guarded against, one must still take care as to how a negative result is to be interpreted. There are at least three possible errors that may occur in the case of a negative gas determination in a nitrate medium:

(1) It may indicate merely a temporary loss of the ability to produce free nitrogen. Thus, in a collection of about two hundred cultures isolated from soil, the writer originally had 10 cultures that produced sufficient gas in a nitrate agar to cause bubbles and cracks within the medium; but two of these cultures, some time later, proved negative in this particular. One of them, however, recovered its ability to produce gas from nitrate after frequent transfer onto this medium.

(2) The medium employed may not be adapted for the organism in question. Such organisms as those above referred to, for instance, will not grow in ordinary nitrate peptone broth.

(3) Too little nitrogen may be produced to be detected. Although nitrogen is not a very soluble gas, a certain amount is needed to saturate the culture fluid, and until the medium is saturated, (or sometimes supersaturated) bubbles will not be produced. Thus, the organisms above-mentioned, when apparently they had lost their ability to produce gas, may actually have been producing free nitrogen but not in sufficient quantities to detect visually.

Nitrite production

A positive determination of nitrite in a nitrate medium is more reliable than a positive observation of gas, inasmuch as there is probably no other source from which nitrite can be obtained except by the reduction of the nitrate. Negative determinations on the other hand, are subject to various sources of error as follows:

(1) Temporary loss of the ability to reduce. Thus the writer has found in a series of 53 cultures, when inoculated on a medium considered to be quite suitable for their growth that 38 produced

nitrite the first time studied, but 3 of them had lost this power on repetition of the procedure; while of the 15 that were negative the first time 2 showed nitrite when the procedure was repeated.

(2) The medium may not be suitable for the purpose, in the case of the organisms under investigation. Many soil bacteria, for example, that show vigorous nitrate reduction on agar, fail to produce sufficient growth to allow the demonstration of nitrite in any liquid medium that the writer has yet investigated. The fluorescent pseudomonads grow well in liquid media, but certain cultures have been found which produce nitrite only in the absence of nitrogenous material other than the nitrate; peptone in these cases seem to have a nitrate-sparing action. The colon organism, on the other hand, has sometimes been found to require at least 0.5 per cent peptone in a liquid medium in order to grow sufficiently to have any action at all on the nitrate added. It is quite obvious in such cases as these that absence of nitrite does not necessarily mean non-reduction of the nitrate.

(3) The reduction may be so rapid that nitrite does not accumulate. With many species of nitrate-reducing bacteria, nitrite is not the end-product, as the reduction may go further, ending with ammonia or free nitrogen. (*a*) In case the nitrite is converted into ammonia as rapidly as it is formed, the situation is a particularly difficult one. It is not difficult to demonstrate ammonia in the culture, but in most culture media there are other sources of ammonia besides the nitrate, so that its presence as an end-product does not necessarily mean nitrate reduction. Difficult as it is to determine this point, however, the writer is convinced that some of the bacteria he has studied have produced ammonia so rapidly from the nitrite that the latter has not accumulated (if they have not in some way converted nitrate directly into ammonia). Evidence of ammonia production has, in fact, been obtained with several different kinds of soil bacteria, some spore-formers and some non-spore-formers, when growing in media containing no nitrogen except potassium nitrate, and some of them have at times been found to give a positive nitrite reaction if tested when only a day or two old, while subsequently showing a negative nitrite reaction. (*b*) When nitrite fails to accumulate

because of the rapid conversion into free nitrogen, the circumstance is not ordinarily so difficult to determine. The writer has observed frequent instances where nitrite could be detected about 24 hours after inoculation, after which gas production would be much in evidence without nitrite showing. It is regarded as rather unlikely that such rapid conversion of nitrite into free nitrogen could occur without visible evidence of gas. This does remain, however, a theoretical source of error that must be taken into account.

(4) Lastly, assimilation of the nitrite by the bacteria must not be overlooked. Undoubtedly, many bacteria convert nitrate directly into cell nitrogen; there are apparently others which reduce nitrate to nitrite as a preliminary step in its utilization. This would seem to be the probable explanation of the fact, above noted, that in the case of certain fluorescent bacteria other sources of nitrogen seem to have the effect of preventing the reduction of nitrate. In case, therefore, nitrate is converted into nitrite in order to be assimilated, it is entirely possible that its conversion into cell nitrogen may be so rapid that nitrite will not accumulate in detectable quantities. Nevertheless, if nitrite is produced as a preliminary step to assimilation, one can hardly say that such an organism does not reduce nitrate. This, accordingly, is another source of error that must not be overlooked.

PROCEDURES AVAILABLE

Before discussing the question of tests to employ in studying utilization of nitrates it must be remarked that anyone studying this point in regard to any culture must first decide the object of his study—whether he merely wishes to make a test for diagnostic purposes in identifying his culture with some published description, or desires to know just what the organism actually does to nitrate. In the former case, he must remember that a statement in some published description that an organism does or does not reduce nitrate ordinarily means that it does or does not produce nitrite in a nitrate medium. For diagnostic purposes, therefore, he can ordinarily get along with a nitrite determination alone; but before attaching significance to the results must decide (by

inference, if no definite statement is available) the conditions under which the author of the published description carried out the test.

If, on the other hand, the investigator desires to know the real action of his culture on nitrate, the problem is much more complicated and a series of tests is often needed, since a negative result is meaningless until supported by evidence from other tests.

The procedures for determining nitrate reduction here given are in no sense new. They have been devised by various authors and have nearly all been compiled in recent editions of the Manual of Methods for Pure Culture Study of Bacteria (Soc. of Amer. Bacteriologists, 1923–35). For this reason the writer has hesitated to publish them under his own name and does so now only by request of certain members of the Committee on Bacteriological Technic. It has, in fact, been pointed out that the discussion concerning nitrate reduction in the above-mentioned Manual is unnecessarily long and involved because there is no definite statement elsewhere in the literature explaining the logic of the procedures for determining this point. Since the writer has always been primarily responsible for the methods on nitrate reduction given in the Manual, other members of the Committee have pointed out to him that it might well help to prevent further confusion if a note on this subject alone were published in a source generally available to bacteriologists; hence the reason for this paper.

Routine test for nitrite. There are, as explained above, many cases where a determination of nitrite alone is of more diagnostic importance than a series of tests to show whether nitrate reduction has actually occurred. The standard naphthylamine-sulphanilic acid test is recommended for this purpose (see Am. Pub. Health Assoc., 1933, p. 19). If the most suitable medium for the organism is not known, one should employ several nitrate media before accepting a negative result. In case such a test alone is made, one should state results not as indicating reduction or non-reduction of nitrate, but as showing production or non-production of nitrite in a nitrate medium.

To overcome attenuation. In case a more complete study of

nitrate reduction is to be made in regard to an organism that produces neither nitrite nor gas on the media used in the above routine test, the first step should always be to determine whether or not the negative results are caused by temporary loss of ability to reduce nitrate. Usually if this is the case, the power can be recovered by repeated transfer upon a nitrate medium on which the organism makes vigorous growth.

To secure a suitable medium. To overcome the error due to a possibly unsuitable medium, no definite rules can be given that apply in all cases. A few general rules are, however, useful for guidance. If the organism is one that is accustomed to grow in the presence of considerable organic matter, the peptone content of the standard medium should be increased or other substances of organic nature added. In the opposite case, the peptone may well be replaced by some inorganic form of nitrogen. In the case of strictly aerobic organisms, agar slants should be substituted for liquid media; while in the case of microaerophilic organisms, semi-solid agar is always worth trying. In short, the investigator must use his ingenuity.

To test for rapid reduction when nitrite does not accumulate. In the case of persistent negative nitrite determinations on all nitrate media investigated, one should always consider the possibility of reduction without accumulation of nitrite. In such cases either ammonia or free nitrogen should be produced. The production of free nitrogen can ordinarily be detected visually, but that of ammonia is more difficult to determine. The Thomas test for ammonia can always be made by the method described in the Manual of Methods (see Leaflet VI, p. VI_{35}–12), but this is of no significance if the medium contains peptone or any source of ammonia other than the nitrate itself, unless the organism can be shown to produce no ammonia from the other nitrogen source.

If, therefore, the organism will not grow with nitrate as the sole source of nitrogen, still further investigation is necessary to show whether or not reduction has taken place. In case the problem is important enough to deserve extensive research, quantitative determinations of residual nitrate in the medium can always be made; but there are also two qualitative tests which

are simple enough to use in routine study and which give valuable indications. First of these is the zinc dust test for residual nitrate as suggested by ZoBell (1932, p. 274). If this proves negative, it certainly indicates that the nitrate has been utilized, and probably reduced, in spite of the failure of nitrite to accumulate. Secondly, it is possible, as suggested by Bronfenbrenner and Schlesinger (1920), to inoculate the organism into a medium containing only a few parts per million of potassium nitrite and then after a considerable period of incubation to observe whether or not the nitrite has been consumed. If it has not been consumed, one can safely conclude that the organism would not be capable of destroying nitrite as rapidly as produced and is not, therefore, a nitrate reducer. If it is consumed, on the other hand, nitrite may have been produced on the nitrate medium without accumulating. Hence, in this latter instance the tests still remain inconclusive.

Concerning assimilation of nitrate or nitrite. In case of the complete disappearance of nitrate without the appearance of nitrite, or in cases where nitrite disappears in the Bronfenbrenner and Schlesinger test, one must be careful to remember that these tests may indicate assimilation (i.e., conversion into cell nitrogen) and not reduction. This point apparently is frequently overlooked.

Incompleteness of the procedures. Even with the various checks and counterchecks proposed in the above system of determining nitrate reduction, there are several possibilities that cannot be definitely accounted for. These are as follows:

(1) Organisms which convert nitrite into cell nitrogen as rapidly as the former is produced by the reduction of the nitrate. (2) Organisms that will not grow with nitrate as the sole source of nitrogen, but which, on a suitable medium, reduce nitrate to ammonia without accumulation of nitrite and produce ammonia from any nitrogen source which permits their growth. (3) Organisms which assimilate nitrate directly cannot, by any methods yet devised, be distinguished from those which reduce it to ammonia without accumulation of nitrite unless they are able to grow on or in a medium with no nitrogen except nitrate.

RECOMMENDATIONS

The chief recommendation of the writer in regard to nitrate reduction is that, for diagnostic purposes, authors of bacterial descriptions confine themselves to the following statement: "Nitrite produced (or not produced) from nitrate on .... medium in .... days." Such a statement is not misleading, and is probably as valuable for diagnosis as one based on a painstaking determination of the end products in such a medium, designed to show whether or not nitrate reduction has actually taken place.

If the author of such a species desires to give a little further information, the following statement may be added: "Nitrate completely consumed (or not consumed) after .... days."

Both of the above statements are based on simple tests that can easily be employed, even on a long series of cultures studied simultaneously A positive result in either case indicates nitrate reduction; and although a negative result does not indicate non-reduction, it is not misleading when stated as above.

Under ordinary conditions the two statements just given are probably enough for diagnostic purposes. If, however, anyone wishes to make a more intensive study and to learn whether an organism that is negative to the two above-mentioned tests is a reducer or non-reducer of nitrate, the further determinations outlined in the body of this paper and given in the Manual of Methods for Pure Culture Study of Bacteria (Soc. of Amer. Bacteriologists) should be followed. It is strongly recommended that not until this has been done should an author of a species publish a statement that it reduces or does not reduce nitrate.

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THE EFFECT OF ELECTROLYTES PRESENT IN THE GROWTH MEDIA UPON THE ELECTROPHORETIC MOBILITY OF ESCHERICHIA COLI[1]

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I. INTRODUCTION

A number of articles have been published (Winslow, *et al.* 1923, 1926) in which the effects of electrolytes upon the electrophoretic mobility of bacteria have been reported. In these studies the organisms were grown, separated from the growth medium, suspended in the electrolyte solution under investigation, and the velocity of migration determined. The presence of low concentrations of electrolytes increased the mobility while higher concentrations decreased the mobility. Analogous results for non-living adsorbents are well known.

Would similar results be obtained if bacteria were grown in the presence of electrolytes or would the bacterial metabolism exercise some control over the mobility? This study attempts to determine this point and is of importance in the discussion of possible relationships between electrophoretic mobility and any bacterial property or activity.

II. EXPERIMENTAL PROCEDURE

A. The suspension. *Escherichia coli* was grown in a 2 per cent aqueous solution of Bacto-Peptone to which had been added the

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[2] Taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Pennsylvania State College.

amount of salt necessary to give the desired concentration. The cultures were kept overnight at 37°C. and centrifuged for one hour. The supernatant liquid was poured off, the cells thoroughly mixed with distilled water, and again centrifuged. After the desired number of washings the bacteria were suspended in a small volume of distilled water, filtered through a small washed plug of cotton, and placed in a refrigerator. Before use each suspension was diluted with distilled water to the concentration most desirable for electrophoretic readings.

B. The determination of mobility. The mobility was determined with a Northrop-Kunitz micro-cataphoresis cell under a potential gradient of approximately 8.8 volts per centimeter. The microscope was fitted with a dark-field condenser and gave a magnification of 460 diameters.

The readings were made in the upper half of the cell at that depth (0.211) which gives actual electrophoretic velocities. For each sample the cell was filled twice, the microscope focused five times and 20 stopwatch readings taken at each focus. The average of these 100 readings was calculated to micra per second per volt per centimeter.

Control determinations were made with untreated bacteria at the beginning and at the end of each set of samples. Because of the excellent agreement between values obtained for the two controls, however, the second came to be considered unnecessary.

C. Washing of bacteria. A study was made of the effect upon the electrophoretic mobility of washing the organisms with water. This was done with bacteria grown in media containing no added electrolyte (blanks) and in media containing $CaCl_2$ and Na_2SO_4. Except in this study all cultures were washed once.

D. Growing time. To ascertain the precision with which the growing time would have to be controlled, an investigation of the mobility of organisms varying in age from 3 to 35 hours was made. All other cultures were grown approximately for 22 hours.

E. pH determinations. These were made colorimetrically using chlorophenol red.

III. EXPERIMENTAL RESULTS AND DISCUSSION

A. Washing studies. Organisms centrifuged from the growth medium and suspended in water were considered to have had "zero" washing. Other samples were washed 1, 2, 3, and 4 times. The results are given in table 1 and the averages in figure 1.

The values for the blanks are practically constant for 1, 2, 3, and 4 washings. For subsequent investigation, therefore, one washing was adopted as the standard procedure.

TABLE 1

Effect of washing upon electrophoretic mobility

	NUMBER OF WASHINGS				
	0	1	2	3	4
Blanks	1.66	2.23	2.23	2.18	2.34
	1.82	2.16	2.22	2.25	2.22
Average	1.74	2.20	2.22	2.21	2.28
$CaCl_2$ 0.01 molar	0.94	1.86	2.05	2.27	2.24
	0.96	1.60	1.81	2.05	2.20
Average	0.95	1.73	1.93	2.16	2.22
Na_2SO_4 0.5 molar	2.42	2.75	2.44	2.32	2.29
	2.38	2.61	2.48	2.36	2.32
Average	2.40	2.68	2.46	2.34	2.31

Each determined value represents the average of 100 readings expressed in μ/second per volt/centimeter.

It appears from figure 1 that in the growth media the mobilities of the bacteria were less than those observed for "zero" washing. It also appears that one washing removes the electrolytes in the case of the blank but not where $CaCl_2$ or Na_2SO_4 were used. If the calcium and the sulphate ions were preferentially adsorbed, their gradual removal by washing would explain the difference in behavior noted for $CaCl_2$ and Na_2SO_4.

The fact that the mobilities eventually obtained, through continued washing of the treated and untreated bacteria, were prac-

tically identical indicates that the salts had produced no irreversible change in the nature of the bacterial surface.

B. Growing time. Possible changes in electrophoretic mobility due to differences in the growing time were sought with media

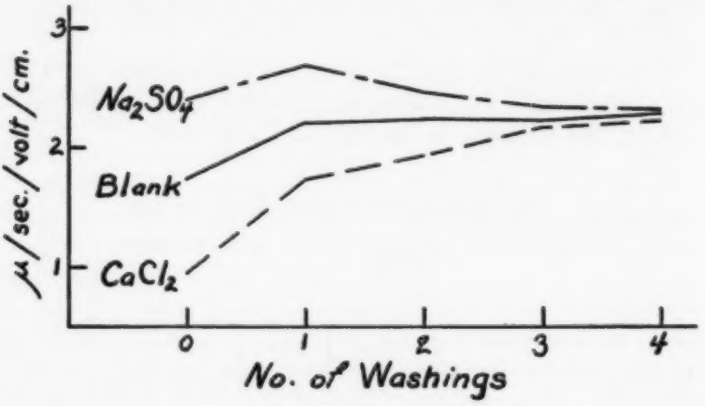

FIG. 1

TABLE 2

Relation of growing time to electrophoretic mobility

	3 HOURS	6 HOURS	10 HOURS	15 HOURS	21 HOURS	28 HOURS	35 HOURS
Blanks	2.29	2.22	2.22	2.26	2.27	2.26	
	2.20	2.22	2.25	2.26	2.24		2.26
Average	2.25	2.22	2.23	2.26	2.26	2.26	2.26
Na_2HPO_4 0.1 molar	1.73	1.61		1.73	1.87	1.90	
	1.70	1.69	1.85	1.84	1.89	1.96	
Average	1.72	1.65	1.85	1.78	1.88	1.93	

Each determined value represents the average of 100 readings expressed in μ/second per volt/centimeter.

containing no added electrolyte and with media 0.1 molar with respect to Na_2HPO_4. The results are given in table 2.

The results for blanks show no change with growing time. Although the Na_2HPO_4-containing media produced a slight up-

ward trend in migration velocity, the change is hardly significant when compared with the data reported by Shibley (1924) for a Type 1 *pneumococcus* and for *Salmonella paratyphi* B. He reported an increase in mobility to a maximum at about 10 hours followed by a decrease to a constant value after about 15 hours. Shibley's findings are apparently not of general applicability. Kahn and Schwarzkopf (1931) working with the tubercle bacillus found that younger cultures have a higher mobility than older cultures. The ages of their cultures varied, however, from four days to five weeks so that the results are not strictly comparable.

The increased mobility obtained might have been due to a decrease in the active concentration of Na_2HPO_4 brought about by bacterial metabolism rather than to any direct effect of the age of the culture.

C. pH values. It has not been possible to correlate the pH values secured with any changes in electrophoretic mobility. The pH values varied, as would be expected, with the multiplication of the bacteria; old cultures showed greater changes from the original than young cultures, and luxuriant growth produced greater changes than meager growth. After the growth of the bacteria the values were all on the alkaline side of neutrality with the maximum at pH 8.4. The data of Winslow and Upton (1926) show practically constant migration velocities for coli from pH 6.0 to pH 8.5 and the values obtained all fall within this pH range. Accordingly it is concluded that changes in migration velocity were not produced by changes in pH.

D. Salt studies. 1. Blanks. The results obtained for the blanks (read before the treated samples) show as an average of 2,740 stopwatch readings a migration velocity of 2.29 μ/seconds for unit potential gradient. The second blank (read after the treated samples) gave the same value for the average of 1,240 individual readings. Of the 62 blanks determined only one showed a greater deviation than ± 5 per cent from the average value of 2.29 μ/seconds per volt/centimeter. This one value was 6.6 per cent too low. The conclusions are therefore drawn that the reading technic is adequate to give reproducible results, that differences in the data are not due to the reading

TABLE 3

Effect of salts on electrophoretic mobility

SALT	CONCENTRATION								CHANGE FROM BLANK AVERAGE 2.29
	0.00001M	0.0001M	0.001M	0.01M	0.05M	0.1M	0.5M	1.0M	
Na_2HPO_4		2.11	2.37	2.22	1.94	1.76	1.33		
		2.14	2.23	2.15	1.85	1.52	1.33		
		2.35	2.36	2.36	1.90	1.76			
		2.34	2.33	2.32	1.88	1.80			
Average...		2.24	2.32	2.26	1.89	1.71	1.33		Decrease
$CaCl_2$	2.23	2.17	2.14	1.83	1.76	1.76			
	2.25	2.13	2.13	1.82	1.52	1.52			
		2.15	2.09	1.79	1.34	1.32			
					1.33	1.65			
					1.75	1.71			
					1.74	1.76			
Average...	2.24	2.15	2.12	1.81	1.57	1.62			Decrease
NaCl		2.24	2.03	2.22	2.18	2.24	2.49	2.56	
		2.25	2.27	2.27	2.46	2.53	2.54	3.19	
			2.37		2.64	2.70	2.58	2.75	
					2.62	2.59	2.59		
Average...		2.25	2.22	2.25	2.48	2.51	2.55	2.83	Increase
Na_2SO_4		2.31	2.21	2.25	2.47	2.67	2.67	3.00	
		2.31	2.23	2.37	2.42	2.55	2.69	2.79	
						2.80	2.69		
						2.52	2.58		
Average...		2.31	2.22	2.31	2.45	2.63	2.66	2.90	Increase
NaI			2.29	2.24	2.17	2.13	2.15*		
			2.27	2.23	2.21	2.14	2.23*		
				2.24	2.26	2.28			
Average...			2.28	2.24	2.21	2.18	2.19*		Slight decrease

* 0.25M.

Each determined value represents the average of 100 readings expressed in μ/second per volt/centimeter.

technic, and that the organism can be grown over a long period of time without change in zeta potential.

2. Effect of salts. The results are given in table 3 and figure 2. The lowest values obtained (0.5 M Na_2HPO_4) represent a decrease in migration velocity of 41.9 per cent; the highest value (1.0 M Na_2SO_4), an increase of 26.5 per cent.

The results for the individual samples receiving the same treatment show in most cases a fairly good agreement one with

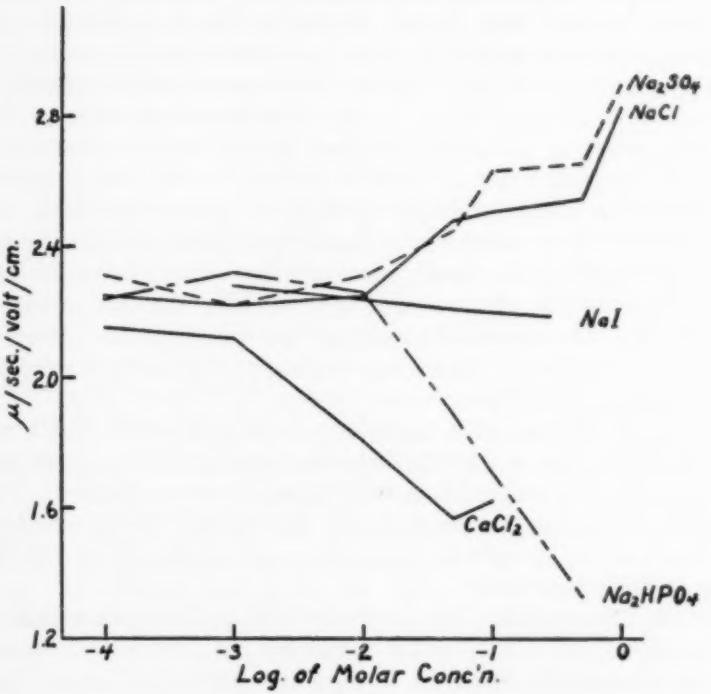

FIG. 2

another, but in a few instances, such as the higher concentrations of $CaCl_2$, considerable differences are evident. Attempts were made to correlate the different values with the age of the culture, the age of the suspension, the age of the distilled water used, and the pH of the growth medium, but no relationship could be established.

No significant change in mobility has been accomplished by the addition of NaI to the growth medium. It is difficult to believe that this salt had no effect upon the mobility of the colon bacilli, but what did happen is not clear.

E. General discussion. Electrophoretic potential varies directly with the thickness of the electrical double layer and with the density of the charge on the particle. Abramson and Müller (1933) indicate that in low concentrations of electrolytes the charge per unit area is the more important factor in determining electrokinetic behavior, while in higher concentrations the thickness of the double layer is the more important factor. The explanation of the results obtained in the washing experiments seems to depend on both of these factors. In the various growth media the thickness of the double layer was very probably considerably decreased and the immediate result of replacing the growth medium by water (zero washing) should be an increase in the thickness of the double layer with a resultant increase in mobility. In subsequent washings the density of the charge on the particles seems to be the factor largely responsible for further changes in mobility.

From the increased mobility encountered with NaCl and Na_2SO_4 it appears that the high concentrations of these two salts in the growth media produce an increased charge density. This high charge density together with the increased thickness of the double layer, brought about by washing, would produce the high mobilities recorded.

The decreased mobility produced with such dissimilar salts as Na_2HPO_4 and $CaCl_2$ would seemingly be due to a decreased charge density. With the $CaCl_2$ a decrease in the negativity of the charge on the bacterial surface undoubtedly occurred, but whether the Na_2HPO_4 acted in a similar manner is not evident even though similar results were noted.

To explain adequately the differences found among the various salts used would require a knowledge of the relative adsorbability of the ions involved and also the active concentrations of these ions in the suspensions studied. In view of these considerations it is not surprising that the data presented do not follow the curves mentioned in the introduction.

The results obtained in this investigation raise a question as to the validity of many of the conclusions arrived at in bacterial electrophoresis. It has been a common practice to suspend washed bacteria in water and to determine the migration velocity of the organisms. From such data conclusions are drawn and classifications are made in attempts to correlate virulence, age of culture, strain of organism, etc., with electrophoretic mobility. The data accumulated here indicate the improbability of any direct relationship existing between the mobility of bacteria suspended in water and the mobility of these same bacteria in their normal environment.

IV. SUMMARY

A study was made to ascertain the effects upon the electrophoretic mobility of *Escherichia coli* of the presence of electrolytes in the growth media. The following conclusions seem justified.

1. The washing with water of colon bacilli grown in peptone broth and in peptone broth containing $CaCl_2$ increases the electrophoretic migration velocity to a constant value.

2. The washing with water of colon bacilli grown in peptone broth containing Na_2SO_4 first increases and then decreases the migration velocity to the same constant value mentioned in (1).

3. Contrary to results reported for other bacteria, the electrophoretic mobility of colon bacilli is not greatly influenced by the age of the culture up to 28 hours.

4. The technic used gives, for colon bacilli cultured over a considerable period of time in peptone solution, average values reproducible within ± 5 per cent.

5. The pH changes which took place did not change the electrophoretic mobility.

6. Under the conditions of this experiment, the electrophoretic mobility can be increased or decreased by the presence of certain salts in the growth medium.

7. The well known mobility-concentration curves determined with salts present in the suspension media were not obtained in this study.

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CONCERNING THE NATURE OF THE GLOBOID BODIES

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Since the causative agent of poliomyelitis is definitely regarded as a filter-passing virus, it is of interest to know the nature of the globoid bodies, which were first reported by Flexner and Noguchi in 1913. These investigators cultivated the virus in a liquid medium of human ascitic fluid containing a fragment of sterile rabbit's kidney under anaerobic conditions. They described a visible growth, after five days' incubation at 37°C., in the form of a gradually diffusing opalescence originating about the tissue fragments. After a third generation, cultivation could be carried on in a semi-solid medium (2 per cent agar added to the above liquid medium), in which minute colonies formed, with globoid bodies arranged in pairs, chains, and small masses (varying from 0.15 to 0.3 micron in diameter). The bodies proved pathogenic to monkeys upon inoculation, even to the eighteenth to twentieth generation (an approximate dilution of the original virus of $1:24^{17}$).

Although many investigators have obtained the same results, the general opinion is that the bodies have a doubtful relationship to the etiological agent of the disease.

EXPERIMENTAL WORK

The initial strain of poliomyelitis virus used in this work was obtained from Dr. J. A. Kolmer, of the Temple University Medical School. All virus cultures were prepared with fresh spinal cords obtained from monkeys with typical experimental poliomyelitis.

The first series of virus cultures was prepared according to the technique described by the original investigators (Flexner and

Noguchi, 1913). The cultural, morphological and staining reactions of such cultures were typical of their descriptions. In addi-

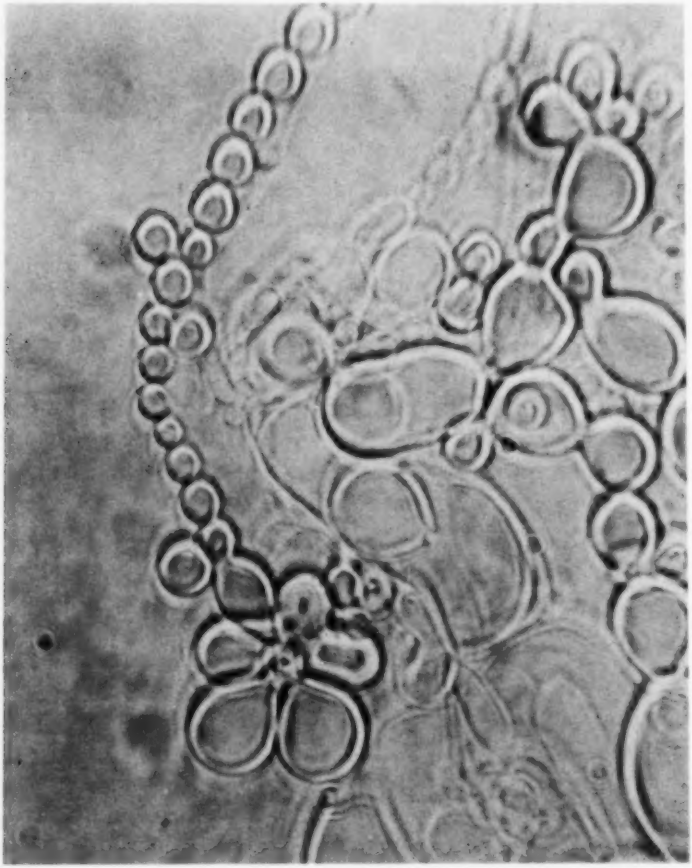

FIG. 1. CHAIN OF SPHERES FROM NEUROFIBRIL. MAGNIFICATION 1800 ×

tion, there were observed in hanging drop preparations from such culture tubes many bizarre forms not described in the literature.

The fanciful forms referred to resembled yeast-like cells budding from mold-like filaments. The filaments are neurofibrils beyond

doubt and the budding yeast-like cells are lipoidic globules which pinch off from such filaments. Filaments as small as 0.5μ were actually observed bearing as many as eight to ten globules in streptococcoid formation and in the middle of the filament. The larger filaments bore many more globules in similar chains. Fig-

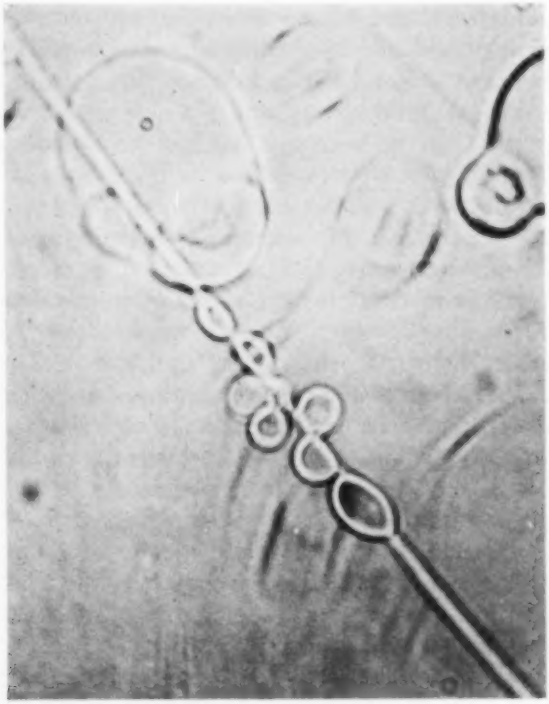

FIG. 2. BUDDING PROCESS IN MIDDLE OF NEUROFIBRIL. MAGNIFICATION 1800 ×

ures 1 and 2 illustrate these distorted neurofibrils. If smaller forms existed, they were too faint to detect in a suspended preparation.

The important distinction between the opalescent tubes and the control tubes is the level at which the globules were found. In the opalescent tubes, the globules migrated to the surface after

pinching off from the filaments at the base of the tube. In the cord control tubes, the globules were restricted to the base of the tube in the immediate surrounding of the tissue and did not migrate to the surface, thus suggesting a physical state permitting an emulsion to occur in the tube containing both virus-infected cord and kidney tissue, and not in the control tubes.

A second series of virus culture tubes was prepared under similar conditions and the following studies were attempted: a measurement of surface tension, determination of the type of emulsion that existed, and staining reactions.

By means of the drop-volume method for measuring surface tension, there was found with increase of opalescence, a lowering of surface tension, thus indicating that "something" was passing into solution and presumably acting as a dispersing agent (Bogue, 1924). The surface tension of the control tubes was lowered a little, but not as markedly as the virus cultures. Although the method for measuring surface tension was rather crude, yet it was accurate enough to indicate that such a physical change occurred with increasing opalescence. Normal sodium hydroxide was used as a comparative standard for consistency in drop-counts per known volume of oil. The volume varied from 10 to 15 drops in a count of 100 drops per cubic centimeter of oil in parallel check tests.

Surface tension of the culture fluid was measured at: (*a*) the beginning of the experiment; (*b*) the sixth day, when opalescence set in; and (*c*) the twelfth day, when sedimentation should have occurred (as described by Flexner and Noguchi).

Comparative estimates of surface tension as indicated by the drop-volume method (drops per 1 cc. of white paraffin oil).

TUBES	(A) INITIAL SURFACE TENSION	(B) 6TH DAY SURFACE TENSION	(C) 12TH DAY SURFACE TENSION
Normal NaOH	101	140	115*
Fluid controls	28	35	31
Kidney controls	27–29	34–38	72–80
Cord controls	27–29	33–37	56–64
Virus cultures	27–29	34–38	108–130

* Range 100–130.

At (*a*) the drop-count agreed as a comparative standard. At (*b*) the drop-count increased, thus indicating a lowering of the surface tension. At (*c*) the drop-count was greater than at (*b*) indicating a still lower surface tension.

The oil-in-water type of emulsion was found. By use of Sudan III as a stain, all sizes of oil globules were observed in the dispersed phase, even as low as one micron. The addition of HCl not only removed the opalescence in the tube but caused the oil forms to coalesce, as observed in the hanging drop exposed to HCl vapors. This was not convincing enough to show that the diffusing opalescence was entirely oil-in-water (or an emulsion formation), and that the globoid forms were all of the same source and substance. Therefore, an attempt was made to produce the same opalescence in the absence of the virus and to observe similar globoid forms from such an opalescence.

Normal rabbit cord and kidney fragments, ascitic fluid and paraffin oil were added to tubes that were corked and coated with paraffin at the bottom. These were incubated for three days. Tubes showing any turbidity or signs of growth were discarded. Those apparently sterile were inoculated with a strain of *Clostridium butyricum* (an exceptionally large-size rod) just below the paraffin oil. The sterile tubes were considered as those in which the cord and kidney fragments appeared as originally placed. The object of inoculating such a tube was two-fold: (*a*) to be certain that any subsequent opalescence would be due to the presence of a living form, and (*b*) to distinguish, microscopically, between an actual bacterium and any micro-coccoid forms which might appear together with it. The organism grew as a pellicle at the surface of the medium. Within five days, an opalescence similar to the virus growth appeared about the tissue fragments at the bottom of the tube. Between surface and bottom the fluid medium was clear. The opalescent fluid was removed from the corked end of the tube without disturbing the pellicle and oil layer. The culture was checked for contamination, other than the organism inoculated, upon blood agar plates and only the clostridia were isolated. Forms similar to the globoid bodies were produced in such a culture. They were distinct and easily differentiated from the bacterial forms inoculated.

Another series was prepared to determine the source of such micro-coccoid forms. This was accomplished with the use of Sudan III. Normal rabbit's cord was stained in bulk for forty-eight hours in an alcohol-acetone (50 per cent of each) solution of Sudan III. A piece of the stained cord, first washed in sterile distilled water, was placed in a tube under conditions similar to those of the previous culture. A number of other tubes were prepared with unstained cord fragments, and Sudan III added to the paraffin oil layer. From such a series it was learned that the oil globules in solution were from both sources, the paraffin oil and the tissue lipoids. However, they could be differentiated, when mixed, by the intensity with which they stained. The cord lipoids were minute and stained rather faintly while the paraffin oil globules were large and stained a deep yellow-pink. They can also be differentiated in an unstained mixture: the lipoids are a pale bluish-green while the paraffin oil is bright and colorless. Dried smears from such an emulsion revealed three distinct types of oil globules: minute, pink bodies the size of, and similar to, the globoid bodies; paraffin oil globules not quite approximating the minute size of the globoid bodies; and a greenish-blue type of oil globule.

Since lipoids are known to migrate only in an electric field (Beutner, 1933), an attempt was made to produce such micro-coccoid forms by the production of an electric current and without the presence of any living cells. This was accomplished by the use of a 3-volt battery connected to platinum electrodes in the fluid. The positive electrode was placed a little below the surface of the ascitic fluid and the negative electrode three-fourths of the way down the tube. With the aid of a platinum wire, a Sudan-stained piece of normal rabbit's cord, which had been washed in sterile distilled water, was held in suspension between the electrodes. Another piece was placed at the bottom of the tube. Within eight hours, the medium above the red stained suspended cord, in the direction of the positive pole, was profusely dotted with red particles migrating from it. Below the cord, the medium was normal. In forty-eight hours a red turbidity extended from electrode to electrode, while the liquid above and

below the electrodes maintained the original clear yellow color. The stained cord at the bottom of the tube remained as it was placed. A dried smear of the red turbidity revealed minute, faintly pink bodies, as well as other forms. The forms observed were not less than 0.5μ in size, the majority being 1μ and larger.

The above series of experiments was repeated with nutrient broth in place of ascitic fluid, but with no success. The physiological salt relations of the ascitic fluid has been found to play a major rôle in the development and migration of the myelin filaments. This was demonstrated by the addition of sodium chloride solution to ascitic fluid which disturbed the natural salt relations. It appears as though the concentration of salt prevents both emulsification of the lipoids and the appearance of bizarre forms of the filaments.

The staining reactions of the forms produced from virus cultures were the same as those produced in the absence of the virus. In the virus cultures, the micro-coccoid forms took a light protein stain while the center was refractive. With precaution against any mechanical violence, such as might rub off of any of the dry smear, staining revealed large lipoid globules with the same refractivity as the micro-coccoid forms (supposedly the globoid bodies). Also, many large lipoids would take the Gram stain as solidly as a bacterium. If such large forms are capable of staining with protein stains, then, surely, the smallest forms of the same substance could be easily mistaken for microörganisms. An interesting fact is the inconsistency of the globoid bodies in taking both acid and basic dyes. With Wright's blood stain, some forms took the pink eosin dye while others took methylene blue. With Giemsa's stain, the same held true. With Gram's stain, some were positive while others were negative. This can be explained on the basis of the type of protein enveloping the lipoid globules. The staining reactions of the globoid bodies are inclined to favor Ransden's theory (1928) of emulsification; that a gelatinous film envelopes the oil globules. "The film is the result of adsorption, accompanied by the diminution of surface energy at the oil-water interface." Such a diminution has been demonstrated by the drop-volume method of measuring surface tension.

The most convincing evidence of the source of the globoid bodies is their reduction of osmic acid to metallic osmium, as illustrated in figure 3. Bacterial smears similarly treated do not reduce osmic acid. Both, the globoid bodies and the similar forms produced in the absence of the virus react in the same way to osmic acid. Black specks uniformly distributed throughout the smear are not to be mistaken for the globoid bodies and micro-coccoid forms. A smear of organisms isolated from blood plates of contaminated tubes, did not respond to osmic acid. Smears

FIG. 3. GLOBOID BODIES FROM VIRUS CULTURE; OSMIC ACID STAIN. MAGNIFICATION 2000 ×

of the globoid bodies and the micro-coccoid globules were placed in Fleming's solution for from two to five days. Since osmic acid is slow in penetrating, daily examinations of the smears showed the lipoid forms becoming more solidly black and losing their refractivity. Lipoids are known to react to osmic acid in such a manner. Since bacteria fail to act in the same way, it may be assumed that the globoid bodies and the micro-coccoid forms are the same. If so, the source and nature of the globoid bodies are evident. Also, since forms similar to the globoid bodies can be

produced in the absence of the virus, the possible relation between them and the virus, is that of adsorption.

Although the experiments which have been carried out by the author thus far have not shown the pathogenicity of such cultures, yet, they have indicated a possible source of micro-coccoid forms that exist in such cultures, and established facts in support of a possible relationship between a virulent substance that is said to increase in vitro (Poliomyelitis, 1932) and the microscopic forms described.

SUMMARY

1. The opalescence reported in the poliomyelitis virus culture can be produced in the absence of the virus.

2. The micro-coccoid forms reported in such virus cultures can be produce in the absence of the virus.

3. The establishment of an electric field will cause tissue lipoids to pass into suspension and react to stains similarly to forms observed in virus cultures. Such forms have been described by other investigators and termed by Flexner and Noguchi as "Globoid Bodies.'

4. A lowering of surface tension was found to take place in the virus cultures as the opalescence increased, thus indicating a physical state which can well account for the stable emulsion that is being interpreted as growth.

5. By the use of osmic acid, minute bodies, 0.2 to 0.5μ in size, are distinctly brought out while the same process has no effect on bacterial smears.

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GRAM-NEGATIVE BACILLI OF THE GENUS BACTEROIDES[1]

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This study was undertaken as an extension of two previous reports on the problem of non-sporulating, anaerobic bacilli; namely, that of Thompson and Beaver, (1931, 1932) and that of Beaver, Henthorne, and Macy (1934). The object of this research was the investigation of the morphologic, biologic, and agglutinative properties of several Gram-negative species of non-sporulating anaerobic bacilli; and a comparison of these species with each other and with the other anaerobes encountered, to demonstrate, if possible, the generic relationships of any or all strains.

HISTORY

Veillon and Zuber (1897) are generally credited with the most important early work on the pathogens of this genus. They reported 25 cases of fetid and gangrenous suppuration, and described various anaerobes as the etiologic agents. One of these anaerobes recovered from osteo-arthritis of an infant was probably

[1] Abstract of thesis submitted by Dr. Henthorne to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Pathology. Read in part before the Society of American Bacteriologists, Chicago, Illinois, December, 1934.

[2] Now residing in Detroit, Michigan.

Bacteroides funduliformis which later was described and named by Hallé. In a more detailed report, which appeared later, these authors (Veillon and Zuber, 1898) said that their research was inspired by the paradoxical observation previously made by Widal and Nobecourt, that pus from gangrenous tissues contained many bacteria, as demonstrated by direct smear, but on culture, either very few organisms grew, or the cultures (aerobic) remained sterile. Veillon and Zuber (1897, 1898) then undertook a systematic research of gangrenous processes with fetid suppurations, namely, pulmonary gangrene, otitis, mastoiditis, abscess of the brain, gangrenous angina, dental caries, appendicitis, peritonitis, peri-intestinal phlegmons, bartholinitis, and pelvic suppurations. They found anaerobes in all of these lesions. Some of these anaerobes were cocci, some were Gram-positive, some were spore-formers, but several Gram-negative members of the genus *Bacteroides* were described, and were found to play an important part in producing the inflammatory processes.

Other authors, notably Rist (1905), Guillemot, Hallé, and Rist (1904) and Wegelius (1909), confirmed the results of Veillon and Zuber by more intensive studies on single organ systems. Aside from systematic research, many reports of cases, in which infections were caused by bacilli of this genus, have appeared in the European literature. Two articles, one by Teissier, Reilly, Rivalier, and Layani (1929), and one by Teissier, Reilly, Rivalier, and Stéfanesco (1931), contributed important clear-cut bacteriologic and clinical data on infections caused by *Bacteroides funduliformis*. These authors reported 4 cases in which septicemia was caused by *Bacteroides funduliformis*, which was diagnosed in pure culture by anaerobic blood culture. They demonstrated agglutinins for *Bacteroides funduliformis* (1 to 800) in the blood of one of their patients. Experiments on complement fixation with their four strains, however, failed to demonstrate close serologic relationship between the strains. These authors also gave an excellent review of the anaerobic literature.

The literature on the genus *Bacteroides* has been less extensive in the United States than in other countries.

In 1901, Norris described a case in which pylephlebitis was

associated with an anaerobic bacillus which, as he recognized, belonged to the group of Veillon and Zuber (*fragilis*?).

Harris (1901–1905) reported a case of pyemia that was caused by a non-spore-forming bacillus which he named *mortiferus*.

Tunnicliff (1913a) reported a motile, Gram-negative anaerobic bacillus (*serpens*?), which she isolated from a case of acute rhinitis. She also reported a case of chronic bronchitis from which she isolated an anaerobic Gram-negative bacillus (1913b).

Oliver and Wherry (1921) described a Gram-negative non-sporulating anaerobic bacillus which utilized hemoglobin for the production of an extracellular melanin or melanin-like substance, which they named *Bacterium melaninogenicum*. They isolated this organism from the pharynx, tonsils, infected surgical wounds, urine, and feces. Burdon (1928) found this same organism in the mouths of normal human beings and animals, as well as in chronic and subacute pathologic processes; that is, pulmonary abscess, and pyorrhea. In cases of puerperal fever, Burdon found *Bacterium melaninogenicum* both in the infected uterus and in the blood stream. It was often accompanied by other bacteria. He considered the presence of *Bacterium melaninogenicum* an index of pollution, and believed that this organism played a prominent part in various pathologic processes, although in pure culture it was not pathogenic for laboratory animals.

Two of us (Thompson and Beaver (1931, 1932)) reported 2 cases of sepsis with formation of abscesses in the lungs. The lesions in 1 case were caused by *Bacteroides funduliformis*; those in the other case, by *Bacteroides fragilis*. We have since expressed the opinion that we were dealing with *Bacteroides funduliformis* in both cases, because both of our strains were hemolytic. We believed that the difference in morphology between the two organisms noted at the time of the report was attributable to the pleomorphism of *Bacteroides funduliformis* rather than to an actual difference of species.

Cohen (1932) reported a review of the literature, and original research on the bacteriology of abscess of the lung. He found a remarkable disregard for the anaerobic flora of abscesses of the lung in previous reports, especially those from the United States.

The anaerobes he encountered were *Bacterium melaninogenicum*, *Bacteroides furcosus*, *Bacteroides thetoides* (*funduliformis*), and *Bacteroides fragilis*, as well as *Fusiformis*, *Leptothrix*, and others. Cohen used the term, *Bacillus*, rather than the generic name, *Bacteroides*.

Two of us (Beaver and Henthorne) with Macy (1934) reported 2 cases of sepsis and hepatic abscess caused by *Bacteroides funduliformis*.

There have been many contributions to the anaerobic bacteriology of stools. The recent one of Eggerth and Gagnon (1933) indicates that anaerobes constitute the predominating organisms in the stool. These authors carried out extensive research on the Gram-negative species of the genus *Bacteroides*, and isolated and studied 118 strains of eighteen species. These strains produced only feeble agglutinins which were strain-specific rather than species-specific. No serologic reactions which could be classed as group reactions were encountered. It is clear then that strict anaerobes which may be classified in the genus *Bacteroides* have been isolated from fetid and gangrenous suppurations of practically all organ systems of the human being, as well as from normal mucous membranes.

PATHOGENICITY

It must be admitted that the *Bacteroides* of feces have not been proved to be pathogenic. The pathogenicity of the species isolated from human lesions, however, including *Bacteroides fragilis* and *Bacteroides funduliformis*, has been demonstrated by various investigators through inoculation of laboratory animals. The mouse has proved relatively refractory to infection especially with *Bacteroides funduliformis*, and the guinea pig has proved more resistant than the rabbit. The latter observation corresponds with our own experience with *Bacteroides funduliformis* in that the four strains previously reported from The Mayo Clinic produced subcutaneous abscesses in guinea pigs, while rabbits often succumbed with systemic infections and abscesses of the liver, lungs, and joints. The two strains described in the second report provided a nucleus about which this research was built and

are listed subsequently as *Bacteriodes funduliformis*, strains 1 and 2.

Unfortunately, we have not had an opportunity to test the pathogenicity of our strains of *Bacteroides fragilis* or *Bacteroides* A for laboratory animals.

CLASSIFICATION

It is apparent that because of its pleomorphism, *Bacteroides funduliformis* has been variously named (*thetoides*, *fragilis*) by different investigators who, after further observation and collaboration, have realized that they were dealing with *Bacteroides funduliformis*. It is possible that other anaerobes, for instance those which have been named *Leptothrix* or *Streptothrix*, are closely related to *Bacteroides funduliformis*. *Actinomyces necrophorus*, also, is similar to *Bacteroides funduliformis* in several important biologic characteristics. This organism is well described by Cunningham (1930), Shaw (1933), and others and is listed by Topley and Wilson (1929) in the genus *Fusiformis* along with *Fusiformis dentium* and pathogenic species of nonspore-bearing anaerobic bacilli, including *Bacteroides fragilis* but not *Bacteroides funduliformis*.

Bacillus mortiferus described by Harris (1901–5) is also similar in most of its biologic reactions to *Bacteroides funduliformis*. One difference exists, that is, *Bacillus mortiferus* formed gas from carbohydrate-free protein mediums. We have not observed this reaction in cultures of *Bacteroides funduliformis*. The nature of Harris' case and the fact that it is the only one reported due to *Bacillus mortiferus* of which we are aware, suggests, however, that he was dealing with one of a closely related group of organisms resembling *Bacteroides funduliformis*.

In general, important biologic characteristics common to all of these variously named organisms are as follows; marked morphologic pleomorphism with production of filaments, requirement of animal tissue in culture mediums, production of indol, production of hydrogen sulphide, production of gas from carbohydrate mediums, and marked hemolytic action on erythrocytes. Whether differences between *Bacteroides funduliformis* and these

otherwise-named non-spore-bearing Gram-negative anaerobes are of major or minor importance must be determined by future comparisons.

Bergey (1934) has classified in the genus *Bacteroides*, several of the fecal bacilli and the pathogens *Bacteroides fragilis* and *Bacteroides fundibuliformis* (it is noted that the spelling of Bergey, with the second syllable "ib" is different from that of the French authors). Among the fecal *Bacteroides*, Bergey has listed several members, which, according to the original describer, do not fit the generic definition, namely, *Bacteroides angulosis*, and *Bacteroides tenuis*, which are described as spore-bearers by Distaso (1911, 1912), and *Bacteroides laevis*, *Bacteroides liquefaciens*, and *Bacteroides rigidus*, which are described by the same author as facultative rather than obligate anaerobes.

ORIGINAL RESEARCH

Material. Gram-negative species of the genus *Bacteroides* were obtained from sources listed in table 1. We encountered three strains of *Bacteroides fragilis* and one strain which we designated *Bacteroides fragilis* 4. Of this group, strain 2 was obtained from blood culture before death as well as from gangrenous lesions cultured at necropsy. Six strains of *Bacteroides funduliformis* were obtained. Of these, strain 4 was isolated from the blood culture taken before death. A strain of another species, probably undescribed heretofore, has been called *Bacteroides* A.

In addition to these species, a strain of *Fusiformis dentium*, one of *Bacteroides bifidus*, an undentified Gram-positive anaerobic bacillus which morphologically resembled the diphtheroid bacilli, and a Gram-negative pleomorphic filamentous organism probably a nonpathogenic Actinomycete have been studied for a comparison with the Gram-negative *Bacteroides*.

Information regarding the lesion cultured, whether or not the organism was recovered directly from the lesion in pure culture, the predisposing factor to bacterial invasion, and the final outcome of the condition is listed in table 1.

Methods. The plating method advised by Spray (1930) was used for determination of the hemolytic action of organisms on blood agar and for isolation of colonies for pure culture.

TABLE 1

Source of strains of anaerobic bacilli

STRAIN	SOURCE	TYPE OF CULTURE	PRIMARY PATHOLOGIC CHANGE	END RESULT
Bacteroides fragilis 1	Pelvic abscess	Mixed	Carcinoma of rectum	Fatal
Bacteroides fragilis 2*	Hepatic abscess and appendix	Mixed	Gangrenous appendicitis	Fatal
Bacteroides fragilis 3	Abscess over sacrum	Mixed	Infected pilonidal cyst	Rapid recovery
Bacteroides fragilis 4	Appendix	Mixed	Gangrenous appendicitis	Fatal
Bacteroides funduliformis 1	Hepatic abscess	Pure	Unknown	Fatal
Bacteroides funduliformis 2	Hepatic abscess	Pure	Carcinoma of rectum	Fatal
Bacteroides funduliformis 3	Hepatic abscess	Pure	Carcinoma of rectum	Fatal
Bacteroides funduliformis 4	Hepatic abscess	Pure	Carcinoma of rectum	Fatal
Bacteroides funduliformis 5	Fecal (?) fistula	Pure	Carcinoma of sigmoid flexure	Recovered
Bacteroides funduliformis 6	Pulmonary abscess	Mixed		Fatal
Bacteroides A*	Appendix	Mixed		
*Bacteroides bifidus**	Hepatic abscess and appendix	Mixed		
Anaerobic diphtheroid*	Hepatic abscess and appendix	Mixed		
Fusiformis dentium	Putrid pleurisy	Pure	Foreign body and pulmonary abscess	Fatal
Actinomyces (?)	Urine†	Mixed		

* Four anaerobes from same case.

† No symptoms referable to bladder.

Strains were maintained with cultures in tubes (20 by 1.5 cm.) of glucose brain broth. All strains grew readily in one or two days. Cultures were also easily preserved in similar tubes of soft agar, to which a piece of fresh brain tissue had been added.

Fermentation was determined in tubes which contained 15 cc. of brain broth; to each tube was added 0.75 cc. of 10 per cent carbohydrate solution which had been sterilized by filtration through a Seitz filter. The marble chips, which usually are added to brain broth as a buffer, were omitted in this procedure. The tubes were inoculated, sealed with semisolid petrolatum, and incubated for two weeks. Production of gas was ascertained by observation of the tubes at frequent intervals, and by the elevation of the petrolatum by gas. The pH value of the culture was estimated at the end of the incubation period by addition of a few drops of bromthymol blue or bromcresol green, and comparison with a standard color chart.

Production of hydrogen sulphide was determined by the action of the gas which was produced by cultures, on filter paper impregnated with lead acetate.

Litmus milk reactions were carried out in the long narrow type of tubes used for brain broth.

Potato cultures were incubated in an anaerobic jar, from which air had been displaced by nitrogen. Pyrogallic acid and a solution of sodium hydroxide were added to remove remaining traces of oxygen.

The difficulty of preparing antigens, especially with *Bacteroides funduliformis*, has been noted by other authors. Growth on slants in anaerobic jars is particularly difficult to emulsify into uniform suspensions because of the tendency of these organisms to grow in long filaments on solid mediums. For this reason, and because growth under these conditions is uncertain, and often feeble, brain broth cultures were used for the preparation of all antigens. Cultures two to four days old were sufficiently well grown for preparation of a satisfactory suspension. The organisms were killed with 1:500 formalin in physiologic saline solution, and were suspended by shaking with glass beads for from five to twenty minutes. The suspensions were finally filtered through cotton and centrifuged at low speed for a few minutes to

remove clumps and particles of brain. The supernatant fluid usually contained a sufficiently dense and homogeneous suspension for the agglutination reactions.

Immune serum was obtained from rabbits which had received injections, at intervals of three to four days, of an antigen which had a density approximately that of a typhoid standard of 500,000,000 per cubic centimeter. Doses were given as follows: 0.1, 0.2, 0.4, 0.8, 1.0, 1.0, and 1.0 cc. The animals were bled one week after the final dose had been administered.

The antigen for absorption of agglutinins was obtained by centrifuging 20 cc. of a suspension of antigen which had a density approximately twice that used for immunization. This bacterial mass was resuspended in 3 cc. of a 10 per cent solution of immune serum in physiologic saline solution. This suspension was incubated at 37°C. for twenty-four hours and placed in the ice box for a similar period.

Morphology. In general, all strains were stained poorly with safranin, but well with dilute carbolfuchsin. Motility was not observed in any strains. Hanging drop preparations proved that the apparent branching, which sometimes was seen in smears, was false. True branching was not observed except in the Actinomycete. In order to obtain organisms for smears or subcultures in broth, it was usually necessary to withdraw material from the bottom of the tube with a capillary pipet.

Two species that fit the original description of *Bacteroides fragilis* by Veillon and Zuber have been isolated in the course of this study. These three strains of one species have been designated *Bacteroides fragilis* 1, 2, and 3. The dissimilar strain, which represents a subspecies, has been designated *Bacteroides fragilis* 4. Morphologically, the two species cannot be distinguished from each other.

The morphology of *Bacteroides fragilis* in fresh cultures and in pus is not especially characteristic. In this form, the bacillus measures 0.5 by 2.0 to 2.5 micra. Older cultures tend to develop larger forms (0.6 to 0.8 by 2.0 to 9.0 micra) but even in this state the organism cannot be distinguished from aerobic Gram-negative bacilli, by its morphology.

Bacteroides funduliformis, on the other hand, is characteristic

morphologically because of its extreme pleomorphism. In the necrotic centers of lesions, growth occurs in such dense clumps of small bacillary forms that it is difficult to pick out the individual bacilli. Bipolar staining is a prominent feature and gives the bacilli a diplococcus-like appearance. Filaments 1.0 by 10.0 micra have been observed in microscopic sections of the granulating periphery of abscesses. On solid mediums, long unbranched filaments develop after six or seven days of cultivation. Fila-

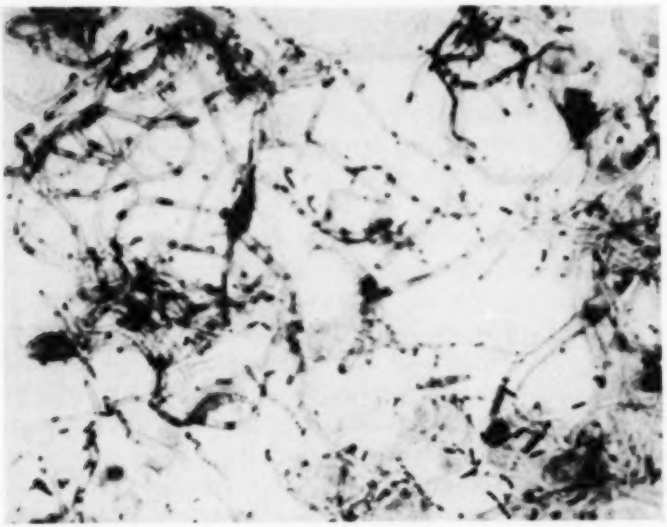

FIG. 1. BACTEROIDES FUNDULIFORMIS FROM TEN-DAY CULTURE ON BLOOD AGAR (×950). STAINED WITH DILUTE CARBOLFUCHSIN

ments of this type are shown in figure 1 (strain 2). These filaments are often 80.0 to 100.0 micra long and are composed of segments 2.0 to 20.0 micra in length. The segments are marked off by granules which are often double. This appearance suggests that the filaments are chains of swollen elongated bacilli and that the granules are the heavily staining beads so frequently seen at the poles of the bacilli. The filaments are 0.6 to 2.5 micra wide and although no "funduliforms" are seen in smears from

solid mediums, there are fusiform swellings 4.0 to 5.0 micra wide. In brain broth the earliest forms are coccobacillary, similar to those seen in pus, or rarely, short curved "comma" shaped rods 0.3 to 0.5 by 2.0 to 4.0 micra. Within two days the characteristic "funduliform," "ball," "leukocyte," or "thetoid" forms appear, together with straight bipolar staining rods which vary in size from coccobacillary up to filaments 20.0 to 40.0 micra long. The ball-shaped organisms (1.5 to 3.0 micra in diameter) seem to be

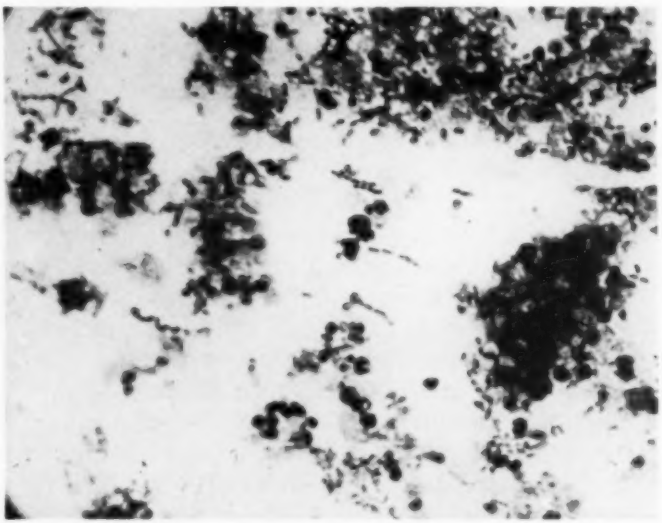

FIG. 2. BACTEROIDES FUNDULIFORMIS FROM TWO-DAY CULTURE IN GLUCOSE BRAIN-BROTH (×1000). STAINED WITH DILUTE CARBOLFUCHSIN

formed by bacilli, swollen so that the bipolar dark staining beads appear as crescentic caps opposite each other on the ball. Figure 2 is made from a smear of a two day glucose-brain broth culture of *Bacteroides funduliformis* (strain 4).

Bacteroides A is slightly shorter and more plump (1.0 by 2.0 micra) than *Bacteroides fragilis*. Bipolar staining in day-old broth cultures is prominent. A characteristic feature of this bacillus is its tendency to develop tremendously swollen forms

even in forty-eight hour cultures. These swollen bacilli are less definite in outline than the "funduliforms" of *Bacteroides funduliformis*, and look like bacteria which are undergoing lysis. Practically all of the bacillary forms show this change at about the same time, but the cultures do not seem particularly delicate, since the bacillus has lived in brain broth for several weeks at room temperature.

The strain of *Bacteroides bifidus* corresponds with the description of Topley and Wilson (1929) of *Lactobacillus bifidus*. Gram-positive granules are present in the bacilli only in very fresh cultures.

The strain of *Fusiformis dentium* was Gram-positive only in the pus from which it was obtained.

The long filaments of the Actinomycete develop in cultures seven or eight days old. Fresher cultures contain only bacillary forms.

The anaerobic diphtheroid loses its diphtheroid appearance when it becomes Gram-negative after three to four days of incubation. Its morphology is not characteristic.

Biologic characteristics. The main biologic characteristics of the group of organisms studied are summarized in table 2. A few points, however, must be further discussed.

In general, all strains have been able to survive in glucose-brain broth for several weeks at room temperature. None of the organisms survive long, however, if exposed to air. They are able to grow in an atmosphere of nitrogen. Although all strains seem to grow better in brain broth which contains fermentable carbohydrate, growth is not improved on solid mediums by the addition of higher concentrations of glucose.

Bacteroides fragilis grows on the surface of plain blood agar as very fine, transparent, moist, grayish white, fimbriated or spreading colonies, without production of hemolysis. Isolated surface colonies do not exceed 2 mm. in diameter. On this medium, growth is seldom perceptible before five days of incubation. Figure 3 is a picture of the surface colonies of *Bacteroides fragilis* on plain blood agar. This picture required special photographic technic because the colonies were so fine and transparent.

TABLE 2

*Biologic reactions of anaerobic bacilli**

	BACTEROIDES FRAGILIS, 1, 2, AND 3	BACTEROIDES FRAGILIS, 4	BACTEROIDES FUNDULIFORMIS, 1, 2, 3, 4, 5, AND 6	BACTEROIDES A	BACTEROIDES BIFIDUS	FUSIFORMIS	ACTINOMYCES
Glucose	++†	++	+‡	++	++	++	++
Maltose	++	++	++	++	++	++	++
Mannitol	−	−	−	−	−	++	−
Lactose	++	++	−	++	++	++	−
Sucrose	++	++	−	++	++	++	−
Levulose	++	++	+	++	++	++	++
Inulin	++	++	−	−	−	+	−
Dextrin	++	++	−	++	−	++	++
Inositol	−	−	−	−	−	+	−
Xylose	++	++	−	−	−	+	++
Raffinose	++	++	−	++	++	+	−
Rhamnose	−	++	−	−	++	++	−
Arabinose	−	++	−	−	−	−	−
Galactose	++	++	+	++	++	++	−
Dulcitol	−	−	−	−	−	−	−
Glycerol	−	−	+	++	−	−	−
Salicin	−	−	−	++	++	++	−
Trehalose	−	++	−	−	++	+	−
Glycogen	++	++	−	−	−	++	−
Sorbitol	−	−	−	−	−	−	−
Production of H_2S	−	−	+§	−	−	−	−
Production of hemolysis	−	−	+++	−	−	−	−
Production of gas on CHO	+ (SI)	+ (SI)	++	++++	−	−	−
Growth on potato	−	−	−	−	−	−	−
Production of indol	∓	∓	+	−	−	−	+
Reduction of nitrates	−	−	−	−	−	−	−
Reaction on litmus milk	Acid, coagulation	Acid, coagulation	−	Acid	Acid	−	−
Liquefaction of gelatine	−	−	−	−	−	−	−

* Anaerobic diphtheroid produced indol, rendered all mediums alkaline; pH of mediums before inoculation was 6.7–6.9.

† ++, pH 4.6–5.0.

‡ +, pH 6.0.

§ In this part of table, positive reactions were graded on basis of + to ++++.

Growth on hormone blood agar is more profuse and more rapid, and the colonies are yellow and opaque. Deep colonies are yel-

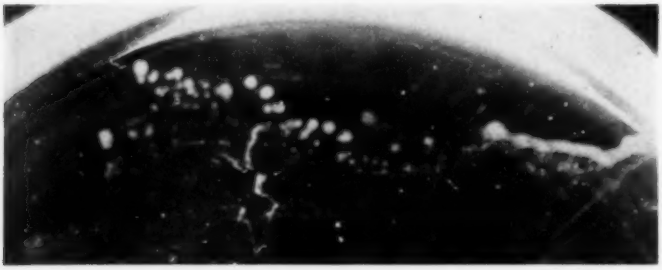

FIG. 3. BACTEROIDES FRAGILIS; SURFACE COLONIES ON BLOOD AGAR

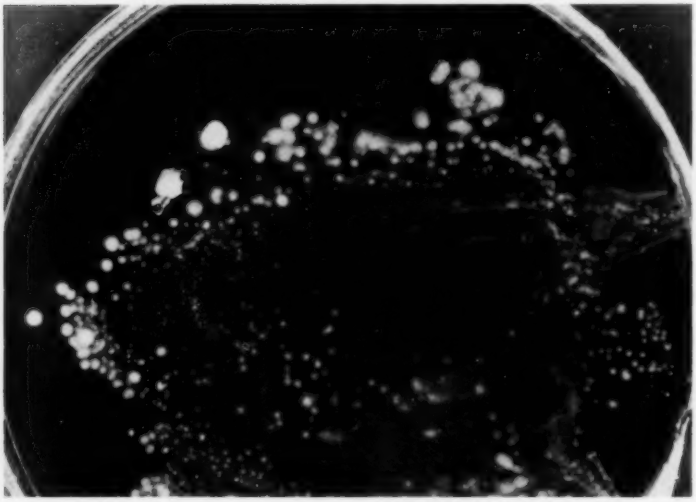

FIG. 4. BACTEROIDES FUNDULIFORMIS; SURFACE COLONIES ON BLOOD AGAR

low, opaque, and lens-shaped. They do not exceed 1 mm. in their longest diameter.

Growth of *Bacteroides fragilis* in glucose-brain broth takes place in twenty-four hours and produces a diffuse clouding of the medium. A characteristic feature of the growth in fermentable

carbohydrate mediums is the slight but definite production of gas. In an anaerobic jar, growth is obtained in peptone-water sugar mediums, but not in plain broth. The biologic reactions of the first three strains are uniformly similar, and those of the fourth strain, which is a subspecies, vary only slightly.

The production of indol by *Bacteroides fragilis* is listed as either negative or positive. For the most part, tests for indol were negative but we have encountered an occasional faintly positive reaction in fourteen-day old cultures.

Bacteroides funduliformis grows on plain blood agar with as much difficulty as does *Bacteroides fragilis*, but the colonies are larger (3 mm.) and more opaque. The centers of the colonies are grayish-white and opaque, the borders are fimbriated and may spread to coalesce with other colonies on a sufficiently moist medium. Figure 4 shows the surface colonies. On slants, when the moisture has drained from the surface, colonies are discoid, discrete, and have slightly raised centers and edges.

The colonies when they first appear are fairly moist, but after incubation for ten days they are hard and dry, and can be picked entire from the medium. Deep colonies are lens-shaped or triangular and measure 2 mm. in their greatest diameters. They occasionally produce gas in blood agar which contains glucose, particularly if the medium is not too stiff. The hemolytic action of this species is an important characteristic. The radius of the zone of hemolysis measures 4 to 5 mm.

In brain broth, *Bacteroides funduliformis* grows in clumps at the bottom of the tube. In fresh cultures, a few bacilli are diffused throughout the broth, but they are scarcely sufficient in number to produce appreciable clouding. Often, the only indication of growth is the presence of bubbles of gas caught in the particles of brain at the bottom of the tube.

The biologic reactions of the six strains of *Bacteroides funduliformis* were very uniform, except that strain 6 did not ferment galactose. This fact was remarkable, since Teissier, Reilly, Rivalier, and Stéfanesco (1931) found their four strains variable. A comparison of the fermentation reactions of their four strains and of the six strains used in this study appears in table 3. The

uniformity of fermentation reactions in our six strains was attributable either to efficiency of the brain-broth carbohydrate medium or to chance, in that strains of only one species were isolated. Very slight reductions of the value for the pH occurred in mannitol and lactose in cultures of the first four strains, but since no production of gas was observed, the fermentation reaction was considered negative.

Bacteroides A differs from both *Bacteroides fragilis* and *Bacteroides funduliformis* in that it grows on plain agar. The colonies are pale yellow and discrete. Surface colonies are 1 mm. in

TABLE 3

*Characteristic fermentation reactions of Bacteroides funduliformis**

	FOUR STRAINS OF TEISSIER, REILLY, RIVALIER, AND STÉFANESCO				SIX STRAINS USED IN PRESENT STUDY					
	1	2	3	4	1	2	3	4	5	6
Glucose	+	++	+	+	+	+	+	+	++	++
Levulose	+	++	+	+	+	+	+	+	++	++
Lactose	−	−	−	−	−	−	−	−	−	−
Sucrose	++	+	−	−	−	−	−	−	−	−
Maltose	++	++	++	++	++	++	++	++	++	+
Mannitol	−	++	++	++	−	−	−	−	−	−
Galactose					+	+	+	+	+	−
Glycerol					+	+	+	+	+	+

* Appreciable amounts of gas formed in all positive reactions.

diameter. Deep colonies produce gas in agar which contains glucose. The most characteristic biologic feature of this organism is its ability to metabolize certain carbohydrates very rapidly with gas production that amounts to a "stormy fermentation." Like *Bacteroides fragilis*, this organism grows in peptone-water sugar mediums but not in plain broth, when incubated in the anaerobic jar.

Agglutination. The agglutination reactions, which are summarized in table 4, support the biologic reactions in proving that *Bacteroides fragilis* 4 belongs to a serologic group which is distinct from the other three strains, and that the six strains of *Bacteroides funduliformis* belong to one and the same species.

The variations recorded in the agglutination of the several strains of *Bacteroides funduliformis* were attributable more to variations in the quality of the antigen than to any marked difference of the strains. As has been mentioned, satisfactory suspensions of this organism were most difficult to obtain in sufficient quantities to use in titrations with all available serums. The results listed in the table are those obtained with relatively large quantities of antigen. Results of agglutination were just as variable in single strains when antigens were later prepared from

TABLE 4

Cross agglutinations

ANTIGEN	SERUM					
	Bacteroides fragilis 1	*Bacteroides fragilis* 4	*Bacteroides funduliformis*	Bacteroides A	*Bacteroides bifidus*	*Fusiformis*
Bacteroides fragilis 1	640	160	80	0	0	0
Bacteroides fragilis 2	640	0	20	0	0	0
Bacteroides fragilis 3	320	80	0	0	0	0
Bacteroides fragilis 4	0	640	0	0	0	0
Bacteroides funduliformis 1	0	0	320	0	0	0
Bacteroides funduliformis 2	0	0	640	40	0	0
Bacteroides funduliformis 3	0	0	640	0	0	0
Bacteroides funduliformis 4	0	40	160	40	0	0
Bacteroides funduliformis 5	0	20	160	40	0	0
Bacteroides funduliformis 6	0	40	80	20	0	0
Bacteroides A	0	0	0	320	0	0
Bacteroides bifidus	0	0	0	0	320	20
Fusiformis	0	0	0	0	0	1,280

time to time. An antigen was prepared from every strain which was agglutinated at 1:320 in the course of the investigation.

Growth of the Actinomycete and the diphtheroid-like bacillus was so sparse, even in glucose-brain broth, that satisfactory antigens could not be prepared either for immunization or for agglutination reactions.

Bacteroides bifidus and *Fusiformis dentium* agglutinated well in their homologous serums, but cross agglutination did not occur with any other strain.

There was some cross agglutination among the Gram-negative species in lower dilutions. These cross agglutinations were not sufficiently definite, however, and not constant enough to demonstrate group agglutinins clearly. The assumption that group agglutinins were not present in the serums was supported by the experiments on the absorption of agglutinins.

TABLE 5

*Absorption of Bacteroides funduliformis agglutinins**

ABSORBING ANTIGEN	TITER
Unabsorbed	320
Bacteroides funduliformis 1	40
Bacteroides funduliformis 2	160
Bacteroides fragilis 1	320
Bacteroides fragilis 4	320
Bacteroides A	320
Control	320

* Bacteroides funduliformis 1 serum and Bacteroides funduliformis 1 antigen were used.

TABLE 6

*Absorption of Bacteroides fragilis agglutinins**

ABSORBING ANTIGEN	TITER
Unabsorbed	1,280
Bacteroides fragilis 1	40
Bacteroides fragilis 2	160
Bacteroides fragilis 4	1,280
Bacteroides funduliformis 1	1,280
Bacteroides A	1,280
Control	1,280

* Bacteroides fragilis 1 serum and Bacteroides fragilis 1 antigen were used.

Normal human serum was titrated with each antigen in all agglutinations, as a control.

Absorption of agglutinins. These experiments may be summarized by reiteration of the fact that agglutinins were not absorbed by antigens prepared from species other than the one against which the animal was immunized. There was, however,

absorption of agglutinins by different strains of the same species. The results of the two most important agglutinin absorption experiments are listed in tables 5 and 6. These two experiments were repeated several times and varied only slightly. When the quantities of absorbing antigens were too small, agglutinins were absorbed only by the antigen homologous with the serum. A second absorption with similar quantities of antigen was necessary under these circumstances in order to demonstrate agglutinin-absorbing properties of various strains of the same species.

The quality of antigens was tested by agglutination before they were used in the absorptions. The control antigen was a suspension of *Escherichia coli*. The immune serum was again controlled by normal human serum.

COMMENT AND SUMMARY

A cultural, morphological and serological study has been made of eleven Gram-negative strains of *Bacteroides* isolated from human inflammatory lesions. Of these, four corresponded closely to the published descriptions of *Bacteroides fragilis*, and six resembled *Bacteroides funduliformis*. We were unable to find a previous description of a strain of another species which we have designated *Bacteroides* A.

Bacteroides fragilis and *Bacteroides funduliformis* are the only species of this genus which we have encountered to which a definite pathogenic rôle can be attributed.

These two species can be differentiated by their morphology and by biologic and serologic tests.

Of the four strains of *Bacteroides fragilis* studied, three seemed identical serologically while the fourth strain belonged to a different serologic group.

The six strains of *Bacteroides funduliformis* fall into one serologic group. We feel, however, that the difficulties attendant on the production of satisfactory antigens make it wise to interpret the results of agglutination tests with caution.

Bacteroides funduliformis has been commonly obtained by us in pure culture from lesions where it is obviously the only pathogen present, and in all but one of the cases in which we have found it, the result has been fatal.

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THE EFFECT OF CERTAIN X-RAYS ON THE ELECTROPHORETIC MOBILITY OF ESCHERICHIA COLI[1]

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Recent researches concerned with the effects of x-rays on bacteria have for the most part considered rates of killing and the theory of action on a sensitive zone. The mechanism of action is not clear, however; certain studies seem to indicate that factors other than direct action on a sensitive zone are involved.

In the elucidation of this problem, changes at the surfaces of the bacteria need to be considered. It is evident that x-rays may affect the distribution of ions at the surface, with a probable resultant change in surface charge and electrokinetic potential. A means of approach to the problem is through the study of electrophoretic mobilities of the particles. Reports of such studies in the literature have been few in number. Lisse and Tittsler (1931) found that irradiation of *Escherichia coli* with ultraviolet light resulted in death with an accompanying decrease in electrophoretic velocity. Dozois *et al.* (1932) reported no change either in viability or in electrophoretic velocity of *Escherichia coli* after irradiation in an aqueous suspension using a Coolidge-type molybdenum-target x-ray tube, the x-rays being

[1] The data in this paper are taken from a thesis submitted by Margaret E. Smith in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Agricultural and Biological Chemistry, The Pennsylvania State College.

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filtered only by the lime-glass window of the tube and the wall of the celluloid vessel containing the suspension. As a general rule, x-rays seem to be lethal to bacteria. In apparent contradiction to the absence of lethal effects noted by Dozois *et al.*, Wyckoff (1930) had found that *Escherichia coli* spread on the surface of nutrient agar were killed by direct exposure to x-rays from a molybdenum target, the only filter being the Lindemann glass window of the tube. With these studies in mind and the object of determining whether death through the agency of x-rays was accompanied by a change in the electrophoretic velocity, the procedure determined upon in the present research was to irradiate the bacteria on the surface of nutrient agar, noting the lethal action, and observing any changes in electrophoretic mobilities resulting from such irradiation.

APPARATUS AND TECHNIQUE

Bacteria. The bacteria used were from a strain of *Escherichia coli* grown on nutrient agar slants. For purposes of experimentation some bacteria were removed from these slants into sterile water and thence to the surface of nutrient agar in Petri dishes, and incubated at 37°C. for 18 to 24 hours. For irradiation the glass cover of the Petri dish was replaced by a lead cover with a hole for the admission of the x-rays. A section of the irradiated portion was used subsequently for the electrophoretic experiments on irradiated bacteria, and a similar section of the part protected by the lead cover was used for the experiments on non-irradiated bacteria, thus ensuring like conditions of growth for the two samples.

X-ray apparatus. Two different x-ray tubes were used: (1) A Coolidge-type water-cooled molybdenum-target tube, having a lime-glass window; (2) a copper target "Phillips Structure Research Tube," the window of this tube being of Lindemann glass. Voltage in each case, supplied by means of alternating current and transformer with the tube itself acting as rectifier, was 30 KV. (root mean square) as determined by a voltmeter-coil on the core of the high voltage transformer; the tube current was maintained at 21 to 22 ma. The distance from the target to the bacteria on

the surface of the agar was approximately 13 cm. By means of the two tubes and the additional use of a filter three types of radiation were produced, theoretically of the following quality: (1) Mo characteristic K radiation together with general radiation of wave lengths longer than 0.3 A (approx.), with the exception that the longer wave lengths were cut out to some extent by the lime-glass window of the tube; (2) Cu characteristic K radiation with general radiation longer than 0.3 A.; (3) essentially Cu Kα radiation, obtained from the copper target tube with the interposition of a nickel filter approximately 0.5 mil. (0.01 mm.) in thickness and two celluloid filters each approximately 1 mil. (0.02 mm.) in thickness. The intensity of radiation from the molybdenum target tube was not determined; intensities from the copper target tube without and with the filters as measured with an open air ionization chamber[2] placed at the level used for irradiation of bacteria were approximately 950 and 440 *r* per minute respectively.

Suspensions. Bacteria, either from an irradiated or from a non-irradiated section of the agar plate, were removed by a small stream of distilled water from a wash-bottle. The suspension was shaken up with glass beads, filtered through cotton to remove clumps, and diluted with distilled water to the desired density for electrophoretic studies. The suspension was stored in a rubber stoppered Pyrex glass flask in the laboratory until use, the end of the experimentation period being not more than three hours after preparation of the suspension. Determinations of pH were made by the LaMotte method using bromthymol blue as an indicator. Since values so obtained were within the range in which Winslow and Upton (1926) found velocity to be independent of pH, and since no correlation was found in the present studies, data concerning the pH of suspensions are not reported.

Electrophoresis cells. A cell of the Northrop-Kunitz type was used in part of the research; the theory and use of such cells has recently been reviewed by Abramson (1934). After sufficient readings had been made at both the upper and lower "stationary

[2] The ionization chamber was kindly loaned by Dr. G. Failla of the Memorial Hospital, New York City.

levels" to make certain that the correct depth was found, readings were made only at the upper level. One hundred observations of velocities were obtained for each experiment, reversing the polarity after each set of 5, refocusing the microscope after 20, and refilling the cell with fresh suspension after 40 or 60 readings. The electrophoretic velocity of a particle is customarily recorded as mobility—i.e., velocity per unit potential gradient. Since the cell used did not have the auxiliary electrodes needed to measure the fall of potential through the cell itself, an estimate of this value was obtained from the dimensions of the apparatus. Hence, mobilities as reported with this cell are in reality only approximate mobilities.

A new "double tube" cell was developed and used for most of the experimental work; the description, theory and advantages of this cell are presented elsewhere (Smith and Lisse, 1936). Briefly, it consists of two tubes in parallel between the electrodes, the dimensions of the tubes having a relation to each other such that no flow of liquid occurs along the axis of the tube of smaller radius, and therefore velocities at that depth are the actual electrophoretic velocities of the particles. The procedure of reversing polarity, refocusing the microscope and refilling with fresh suspension as used in the work with the Northrop-Kunitz cell was followed. In some cases, however, a total of only 60 or 80 readings were taken in one experiment; a smaller number is permissible, since there is less variation in the readings taken with this cell, due to the fact that observations are made at the level where the velocity gradient is minimum. From these observations values designated as actual mobilities were calculated, the potential gradient through the cell being given with sufficient accuracy by E/L, where E is the fall of potential between the electrodes as measured with a voltmeter, and L is the length of the tube of smaller radius.

RESULTS OF EXPERIMENTS ON LETHAL ACTION OF X-RAYS

A series of experiments were carried out primarily to determine whether the bacteria as used in the electrophoresis studies were killed through the action of x-rays. The technique used in most

of the experiments was similar to that used by Wyckoff (1930), the bacteria being spread on the surface of nutrient agar, a section irradiated, and counts made of the number of colonies developing on an irradiated section and on a control section of equal area. Table 1 gives the results of these experiments, each number being the average of percentages obtained from 10 or more counts from irradiated sections and from an equal number of control sections. Conclusions concerning rates of killing cannot be made from the data, due to insufficient number of experiments and also to the fact that voltage and current were not sufficiently well controlled to allow for quantitative results, particularly in the cases of short-

TABLE 1

Percentage of surviving organisms (Esch. coli) after exposure to x-rays of different nature for various periods of time, from ½ to 120 minutes

PERIOD OF IRRADIATION	TYPE OF RADIATION		
	Mo K characteristic + general	Cu K characteristic + general	Cu K_α
minutes	*per cent*	*per cent*	*per cent*
½		48	60
1		36	41
5		2	5
10	38	0	1
20	12	0	0
120	0	0	0
Intensity in *r* per minute.....		950	440

time irradiation. However, allowing for the differences in intensities used, the results are of the same order as those given by Wyckoff.

Five experiments were concerned with the lethal effects of copper characteristic K and general radiation on *Escherichia coli* in aqueous suspension. These showed that while the rays were lethal, the rate of killing was much less than when the bacteria were exposed directly. Since it is apparent (see table 1) that x-rays from the molybdenum target are far less lethal than are those from the copper target, an explanation of the absence of lethal effects noted by Dozois *et al.* (1932) may be that insufficient dosages were given to make the effects observable.

Further experiments showed that irradiation of the medium and subsequent covering of the surface of the agar with *Escherichia coli* did not result in death of the bacteria; and also that the lethal effects of copper radiation are cut off, or at least greatly diminished, by the interposition of the glass cover of the Petri dish between the x-rays and the bacteria.

For the electrophoresis studies, the surface of the agar was covered with a more dense suspension than for the above studies; the bacteria were grown for 24 hours at 37°C. before irradiation, thus being in colony form. Sub-cultures were also made from these irradiated sections and incubated to determine the presence of viable cells. Conclusions from the lethal studies which are of particular interest in the electrophoresis studies are:

1. Twenty-minute exposure of *Escherichia coli* to Cu characteristic K and general radiation, or to Cu K_α radiation resulted in death of all of the bacteria in the irradiated section on an agar plate, either in the case where the bacteria were spread out on the plate, or where colony formation was present.

2. Twenty-minute exposure to Mo characteristic K and general radiation did not result in death of all of the bacteria in the irradiated section.

3. Long-time irradiation, with any of the three types of radiation used, resulted in death of all of the bacteria on the irradiated section.

EXPERIMENTAL RESULTS OF ELECTROPHORESIS STUDIES

Five series of experiments concerned with electrophoretic mobilities of irradiated and non-irradiated bacteria were performed. The irradiated bacteria were suspended in distilled water in the manner previously described; conditions of irradiation for the five series were as follows: in series I, the bacteria were exposed for 20 minutes to radiation from the molybdenum target tube, and in series II to unfiltered radiation from the copper target tube for 20 minutes; in series III and IV, the same two types of radiation were used respectively, but the period of exposure was 120 minutes; series V consisted of 240-minute exposure to Cu K_α radiation.

Each series comprised 10 pairs of experiments, the Northrop-Kunitz cell being used for 8 pairs of experiments in series I and 7 pairs in series II, and the new cell for the remainder. A pair of experiments consisted of the 60 to 100 observations on bacteria taken from the irradiated section of the plate, and also of the same number of observations on the non-irradiated bacteria obtained from a protected portion of the same plate. In each pair of experiments, conditions of growth of bacteria, suspension medium, the cell used and temperature were practically identical. The mobility in the case of the new cell, or the approximate mobility in the case of the Northrop-Kunitz cell corresponding to each observed velocity, was calculated, and the mean mobility—*i. e.*, arithmetic mean (A.M.) of the 60 to 100 values was determined.

Frequency distribution diagrams of the mobilities obtained in the experiments indicated that the data for an experiment tend to conform to the normal distribution curve; it appeared that the calculation of standard deviation[3] (S.D.) might be of value. Two advantages of such determinations were made use of in the accumulation of data. The value obtained serves as a criterion for data which need to be examined critically and perhaps discarded, since an unusually high value might result from errors such as focusing on different levels, or drifting in one direction. Furthermore, use of the standard deviation gives information as to the number of values which should be determined in any experiment for the desired amount of accuracy. Since variation in readings with the new cell tended to be less than with the Northrop-Kunitz cell, a smaller number of observations was justified.

Table 2 gives the values of the mean mobilities and the standard deviations calculated for the 10 pairs of experiments in series I.

The per cent change in mobility resulting from irradiation in series I, calculated from the values of the arithmetic means of the

[3] Standard deviation is defined by:

$$\text{S.D.} = \sqrt{\frac{\Sigma(X - \text{A.M.})^2}{N}}$$

where $X - \text{A.M.}$ is the difference between a value X and the mean, and N is the number of values of X.

mean mobilities for the irradiated and non-irradiated samples (see table 2) is −2.8 per cent. The customary method of treating data of electrophoretic mobilities has been to select a more or less arbitrary per cent change as the limit of experimental error to be allowed. In selecting this value, factors such as the number of experiments performed and the amount of variation of values

TABLE 2

The mobilities of irradiated and non-irradiated Esch. coli in aqueous solutions

Conditions of irradiation: Mo x-rays from Coolidge type tube operating at 30 $KV_{r.m.s.}$, 22 ma., impinging for 20 minutes on bacteria spread on surface of agar, distance 13 cm.

PAIR NUMBER	CELL USED	A.M. AND S.D. OF MOBILITIES IN μ/SEC./VOLT/CM.			
		Non-irradiated		Irradiated	
		A.M.	S.D.	A.M.	S.D.
(1)	(2)	(3)	(4)	(5)	(6)
1	N.K.*	3.23	0.29	2.96	0.31
2	N.K.	3.62	0.35	3.62	0.32
3	N.K.	3.76	0.32	3.78	0.41
4	N.K.	3.75	0.31	3.53	0.35
5	New†	3.70	0.19	3.95	0.35
6	New	4.03	0.23	3.98	0.30
7	N.K.	3.33	0.27	3.39	0.28
8	N.K.	3.34	0.21	3.28	0.21
9	N.K.	3.33	0.31	2.87	0.23
10	N.K.	3.23	0.29	2.96	0.31
Sum.....		35.32		34.32	
A.M.....		3.53		3.43	

* N.K. = Northrop-Kunitz cell.

† New = new type cell.

from the mean should be considered; that is, the use of statistical methods is indicated. In the present study a means of determining whether changes to the extent found are significant was sought through the use of "Student's" method of analysis for paired data. This method is given by Pearson (1914) together with the table of probabilities. Love and Brunson (1924) discuss the applicability of this method, and Love (1924) gives a table

of the "odds," indicating whether values determined are significant.

Analysis of the data given in columns (3) and (5) of table 2 with reference to Love's table shows that the odds are less than 12:1 against the difference in mean mobilities of the non-irradiated and irradiated samples being due to chance alone. Odds greater than 20:1 are usually termed significant; odds greater than 100:1 are very significant. Thus, analysis shows that no significant change in electrophoretic mobility was brought about through irradiation of *Escherichia coli* for 20 minutes with x-rays from the molybdenum target tube. A similar analysis of the data given in columns (4) and (6) of table 2 shows that the odds are

TABLE 3

Summary of changes in mobility and in variation in mobilities of Esch. coli with exposure to x-rays of different wave lengths

SERIES NUMBER	TYPE OF IRRADIATION	INTENSITY	TIME	CHANGE IN MOBILITY	CHANGE IN VARIATION
		r/minute	*minutes*	*per cent*	
I	Mo K + general		20	0*	0
II	Cu K + general	950	20	0	0
III	Mo K + general		120	0	0
IV	Cu K + general	950	120	−15.4	0
V	Cu K_α	440	240	−7.9	0

* 0 indicates that changes were not significant.

less than 10:1 against the differences between the standard deviations of the non-irradiated and irradiated samples being due to chance alone; therefore we would conclude that no significant change in the variation of mobilities has resulted from irradiation.

Analyses by "Student's" method of the data obtained in the other four series show that very significant changes in the mobility resulted from irradiation in the experiments of series IV and V, the odds in these 2 cases being greater than 10,000:1 and 300:1 respectively; in no other cases were the changes significant. Table 3 gives a summary of the results obtained in the electrophoretic studies for the five series. Changes which are not of sufficient amount to be significant are indicated in the table by 0; in the 2 cases where significant changes occurred the per cent of

change, calculated from the values of the arithmetic means of the mean mobilities, is given.

DISCUSSION OF RESULTS

The lethal studies showed that 20-minute exposure to copper characteristic and general x-radiation resulted in death of all of the bacteria in the irradiated section, whereas exposure for the same length of time to molybdenum characteristic and general radiation resulted in death of only a certain percentage of the bacteria. In either case no significant change in electrophoretic mobility was observed. This clearly indicates that change in electrophoretic mobility is not a criterion of death of the organism, or at least that any change produced is not of sufficient magnitude to be observed by the methods used.

A change in mobility can be brought about through the agency of x-rays as is evidenced by the decreased mobility resulting from long-time irradiation either with copper characteristic and general radiation, or with Cu K_α radiation. It is difficult to state with certainty the cause for the decreased mobility. As noted previously, Lisse and Tittsler (1931) found that irradiation of *Escherichia coli* with ultraviolet light resulted in decreased mobility; it is possible that wave lengths in this range are effective in the present case. Such wave lengths would be present in appreciable amounts in the unfiltered copper radiation, and might result from fluorescent radiation of the Cu K_α radiation, and probably to a very small extent from the molybdenum radiation. Experimental evidence is in accord with this view, since the copper general and characteristic radiation was the most effective, and the molybdenum radiation the least effective in changing the mobilities. Another explanation might be that death of the bacteria brings about conditions, such as changes in permeability or adsorption, which may result after some time in the decrease in mobility noted.

From the studies it appears that there is a difference in action of different wave lengths. The dosages of Cu characteristic and general radiation and of Cu K_α in the experiments of series IV and V were 11.4×10^4 and 10.6×10^4 *r* respectively (see table 3

for data), their ratio being approximately 1.1:1; the ratio of resultant changes in velocity was approximately 15:8. With due consideration of the relatively large experimental error inherent in this type of experiment, it appears that the decrease in mobility is a function of the quality of the radiation, not merely of the intensity. Wyckoff (1932) suggested that the lethal effects of x-rays and ultraviolet light might not be the same, even though semi-logarithmic curves of death were found in both cases. The experiments reported here seem to corroborate this view. Thus, while death through the action of ultraviolet light and certain other agencies may be due to action on the exterior of the organism, the lethal effect of x-rays may be due to direct action on a sensitive zone or nucleus.

It is of interest to note that no change in variation of mobilities occurred, even in the 2 cases where the mean mobilities did change. That is, the values of the mobilities of the irradiated samples seem to conform to distribution curves of the same type as those of the non-irradiated samples.

CONCLUSIONS

1. No significant change in electrophoretic mobility of *Escherichia coli* in aqueous suspension resulted from:

(a) Twenty-minute exposure of the bacteria on the surface of nutrient agar to molybdenum characteristic K and general radiation (30 $KV_{r.m.s.}$, 22 ma., 13 cm. distance); this dosage was sufficient to result in death of only a certain per cent of the organisms in the exposed section.

(b) Twenty-minute exposure of the bacteria to copper characteristic K and general radiation. The dosage of 1.9×10^4 *r* was more than sufficient to result in death of all of the irradiated organisms.

(c) One hundred and twenty-minute exposure of the bacteria to the molybdenum characteristic K and general radiation; this dosage was greater than that required to kill all of the organisms in the exposed section.

2. Death through the agency of characteristic and general molybdenum or copper x-radiation is not accompanied by a

change in the electrophoretic mobility of the bacteria in aqueous suspension to an extent measurable by microscopical studies of the type made.

3. Exposure of *Escherichia coli* on the surface of nutrient agar to dosages of Cu characteristic K and general radiation or to Cu K_α radiation in amounts much greater than is required to bring about death of all of the organisms in the exposed section results in a significant decrease in the electrophoretic mobility of the bacteria in aqueous suspension. The Cu characteristic K and general radiation seems to be more effective in this respect than is the Cu K_α radiation.

4. Values of mobilities of irradiated samples seem to give distribution curves of the same type as those of the non-irradiated samples, no increase in the amount of variation being found as a result of irradiation.

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BACTERIAL GROWTH AT CONSTANT pH

QUANTITATIVE STUDIES ON THE PHYSIOLOGY OF LACTOBACILLUS ACIDOPHILUS

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In a recent article the authors (1935) have described the results of an investigation on the acid production of *Lactobacillus acidophilus* at various constant pH values. It was found that increases in acid formation as high as 200 per cent over the yield with no pH control could be obtained by maintaining the pH near the optimum value of about 6. However, it was found necessary, since the medium was stirred, to study the effect of the gas with which it became saturated. One conclusion, in which we confirmed the work of earlier authors, was that carbon dioxide is necessary for the growth of the organism.

Since the effect of dissolved gases on bacterial growth is evidently of importance and interest we have followed the subject further with the results to be described in the present paper. A series of studies of the development of the bacteria were made in which the temperature and the pH were kept constant, and the gas mixtures (of nitrogen, oxygen and carbon dioxide) with which the medium was saturated were varied in composition. In addition to the acid production, population studies were made with the aid of plate counts, turbidity measurements were carried out with a photoelectric turbidometer, and the apparent oxidation reduction potentials (E_h values) were followed.

Some improvements have been made in details of the apparatus for maintaining a constant pH value. These include a device for improving the accuracy of the determination of the acid produced during the early part of the growth period, and a means for

eliminating possible contamination during the introduction of the electrodes into the medium.

The turbidometer measurements will be discussed in a later paper.

EXPERIMENTAL

The modified buret. Since the magnetically operated buret (*B* of figure 1 of the previous article, Longsworth and MacInnes, 1935) had to furnish as much as 400 cc. of normal alkali during the growth period it was made of large-bore tubing. It was, therefore, not possible to read changes in the level of the liquid with the desired accuracy in the early part of the growth, when the amounts of alkali drawn from the buret were small. A simple modification of the buret, shown in figure 1, makes it possible to obtain nearly the same relative accuracy throughout the growth period. The two graduated cylinders *A* and *B* are connected by the rubber tube *R* which can be closed by a pinch cock. The alkali in these cylinders is protected from atmospheric carbon dioxide by means of soda lime tubes connected to *C* and *C'*. Operation of the magnet, attached at the point *M* to the central glass rod *D*, lifts the rubber plug *F* and allows the alkali solution to flow to the culture medium through the capillary tube *E*. If the pinch cock at *R* is closed, the solution is drawn from the graduated tube *B* only, with the result that small volumes of fluid entering the medium can be read with accuracy. When the rate of acid formation approaches its maximum the pinch cock at *R* can be opened so that the alkali solution is drawn from both graduated tubes. As constructed, the cylinders *A* and *B* deliver 17.8 and 1.2 cc. respectively, per centimeter of length.

The apparatus for sterilizing and inserting electrodes. As explained in the previous paper, it is not possible to sterilize the glass electrodes in an autoclave. The electrodes will disintegrate with such treatment, or at least lose their calibrations. It is therefore necessary to resort to chemical sterilization. Although little difficulty was encountered with the method already described for accomplishing this end, there remained a slight possibility of contamination during the short time in which the

electrodes were being inserted into the culture flask. It was therefore considered desirable to construct a device by means of which the electrodes can be sterilized, rinsed and inserted into the medium without coming into contact with laboratory air. The arrangement, which is an adaptation of the Richards "bottling apparatus" (Richards and Parker, 1896), is shown in figure 2. The opening of the flask *F* into which the electrodes are to be

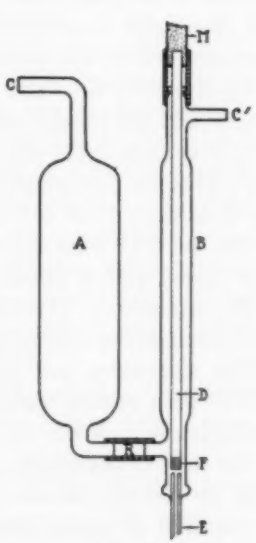

FIG. 1

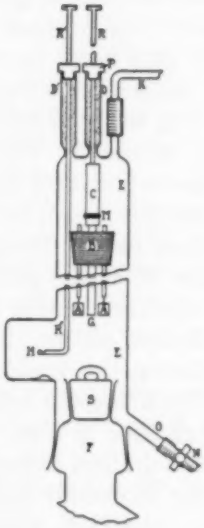

FIG. 2

FIG. 1. THE MODIFIED BURET
FIG. 2. THE APPARATUS USED FOR STERILIZING AND INSERTING ELECTRODES

placed is closed with the glass stopper *S* while the flask and its contents are in the autoclave and so kept until the electrodes are inserted. The glass electrode *G* and the gold electrodes *A*–*A* are mounted on the bakelite stopper *B* and are held in place in the collar *C* by means of the spring clip *M*. The electrodes may be raised and lowered by means of the metal rod *R*. This rod passes through the glass cylinder *D*, which is closely packed with cotton, and through the metal guide *P*. This guide is kept in

place, and maintains a pressure on the cotton, by means of springs (not shown). Another rod R' passes through the glass cylinder D' which is also packed with cotton. With the aid of the hook on the end of the rod R' the stopper S can be lifted out of the flask F and placed out of the way in the recess H, after which the electrodes on the stopper B can be lowered into the flask with the aid of the rod R.

In use the procedure is as follows. The glass tube E–E with its attachments, but without the electrodes, is mounted upon a stand which also holds an aspirator bottle containing water, with which connection is made by the tube K. The bottom of the tube E–E is wrapped with paper and the combination is sent through the autoclave. The paper is then removed, the electrodes are inserted, and the flask F, with the stopper S in place, is put in the position shown in the diagram. Five per cent cresol solution is then run in through the tube O with the aid of air pressure, after which the outlet is closed with a pinch clamp N. The cresol solution remains in the apparatus overnight, after which it is drained off, air filtered through the cotton plugs in D and D' replacing the liquid. The electrodes are then rinsed three times by filling the tube E–E with sterile water which is forced through tube K from the aspirator. After the last rinse water has been drained through the tube O the stopper S can be lifted and the electrodes inserted as already described. After removing the tube E–E, melted vaseline is immediately poured on top of the stopper B.

The measurement of apparent oxidation-reduction potentials. The apparent oxidation-reduction potentials (E_A values) were obtained by measuring the electromotive force between the gold electrodes A–A shown in figure 2 and the 3.5 N calomel half-cell which also served as a reference for the glass electrode. As described in the previous paper, electrolytic connection of the medium with the 3.5 N KCl is made through a dense porous plug. The potentials were obtained with a Compton electrometer using a high resistance potentiometer and a Hulett cell, also described in connection with our earlier work. The gold electrodes consist of sheets of the metal 1 cm. square welded to platinum wires

which are sealed through glass tubing. After welding, a thin film of gold was melted onto the exposed platinum wire.

Procedure for an experiment. After the electrodes are inserted, as described above, in the flask *F* containing the culture medium, the flask is placed in the thermostat operating at 38.0°. Connection is made with the stirrer, the reference calomel electrode, and to the buret by means of which alkali is added to maintain the pH at a constant value by automatic control, as described in the previous article. A stream of gas of the desired composition is passed through the stirred medium. Equilibrium at the chosen pH between the dissolved carbon dioxide and sodium bicarbonate (formed if necessary by the flow of alkali solution from the buret) is slowly established. Measurements of the E_h values during this period indicate that they are approaching a value characteristic of the sterile medium in equilibrium with the gas. Twenty-four hours are allotted for this preliminary equilibration. A valuable test is also furnished by this procedure of the success of the technique in avoiding bacterial contamination since such contamination is indicated by turbidity of the medium and by rapid lowering of the E_h.

The stock culture of *Lactobacillus acidophilus* used in our experiments was carried in litmus milk with frequent subcultures. In order, however, to avoid the introduction of turbid materials from the milk into the growth medium used in the apparatus two subcultures were made in that medium.

THE RESULTS AND THEIR INTERPRETATION

To illustrate the nature and extent of the results obtained during this investigation the data recorded during one experiment are given in table 1. In that table the time given in the first and sixth columns was read from the moment of inoculation. The figures for the acid production in the second column are computed from the buret readings and are stated in milli-equivalents per liter of bacterial suspension. After each buret reading a 25 cc. sample was withdrawn. Portions of this sample were then taken for the plate counts. The plate counts, listed in the fourth column, were obtained in the conventional manner, and are the

mean value of the figures from four plates, the average deviation from the mean being 5.5 per cent. In the intervals between buret readings potential measurements were made from which the E_h values in the last two columns were obtained, using $E_0 = 0.246$ for the potential of the 3.5 N calomel electrode against the normal

TABLE 1

The results obtained in a typical experiment on the growth of L. acidophilus with automatic pH control

Temperature, 38°. pH, 6.0. Medium: 2 per cent peptonized milk; 8 per cent galactose. Gas: CO_2 8.2 per cent; $O_2 < 0.2$ per cent; N_2 91.6 per cent. Titrating fluid: 1.0 N NaOH.

TIME	MILLI-EQUIVALENT ACID		PLATE COUNT		TIME'	Eh_1	Eh_2
	Observed	Corrected	Observed	Corrected			
hours			*10^9/ml.*	*10^9/ml.*	*hours*		
0.0			0.201		−0.08	+0.064	+0.064
2.0			0.550		1.67	0.059	0.060
4.0			1.35		4.22	0.054	0.054
6.0			3.68		5.60	0.047	0.047
8.0	0.0+	0.0+	12.9		7.52	0.023	0.023
10.0	0.5	0.5	38.8		10.35	−0.058	−0.055
12.0	4.4	4.5	124.0	124	11.85	−0.108	−0.101
14.0	12.9	13.3	204	207	13.80	−0.136	−0.127
16.0	26.4	27.4	475	488	15.88	−0.155	−0.147
18.0	49.1	51.7	784	825	17.78	−0.179	−0.178
20.0	71.8	76.6	886	954	19.80	−0.178	−0.179
22.0	90.0	96.7	981	1,076			
24.0	104.8	113.3	995	1,108	23.57	−0.167	−0.170
33.0	154.4	170.3	1,060	1,241	32.80	−0.171	−0.174
48.0	216.6	242.6	904	1,124	47.07	−0.163	−0.167
58.0	249.2	281.3	892	1,144	57.50	−0.155	−0.159
72.0	286.9	326.5	822	1,091	71.25	−0.155	−0.159
83.0	312.3	357.5	761	1,033	82.58	−0.156	−0.159
96.0	337.9	389.2	683	949	95.38	−0.158	−0.161

hydrogen electrode. The agreements of the potential readings from the two gold electrodes are typical of those obtained during this research. The readings agree within 3 mv. except when the potential is changing rapidly, when the difference may become somewhat greater.

In addition to the experiment, No. 6, the data of which are given in table 1, others were carried out using different gas mix-

tures, and a different concentration of galactose. The conditions of these experiments are outlined in table 2.

Since the growth medium is progressively diluted by the addition of the titrating fluid, and samples for examination are periodically withdrawn, the original buret readings and the plate counts are subject to corrections which may be outlined as follows: (*a*) *The buret readings are to be corrected to the values they would have had if no samples were taken.* It is convenient for the derivation to postulate that an inert reference substance A is present to the extent of n_0 moles in the original medium. Dilution with the titrating fluid will not change the amount of this substance, but withdrawal of samples will reduce it. Thus the addition of Δv_0 cubic centimeters of titrating fluid before taking the first

TABLE 2

Conditions of experiments on the growth of L. acidophilus at pH = 6.0 and 38°

EXPERIMENT	DATE	MEDIUM		GAS		GENERATION TIME, "LOGARITHMIC PERIOD"
		Peptonized milk	Galactose	CO_2	O_2	
		per cent	*per cent*	*per cent*	*per cent*	*minutes*
1	11/ 5/34	2	3	8.1	0.3	
2	1/25/35	2	3	8.1	0.2	80
3	3/25/35	2	3	8.2	0.2	76
4	2/23/35	2	3	41.6	0.2	73
5	5/11/35	2	3	7.6	2.1	66
6	3/15/35	2	8	8.2	0.2	74

sample will not change the amount of A and thus Δv_0 needs no correction. However, removal of a sample of s cubic centimeters reduces the amount of A to n_1 in the proportion

$$n_1 = n_0 \frac{V_1 - s}{V_1}$$

in which V_1 is the volume of the growth medium before withdrawal and $V_1 - s$ the volume immediately after. Titration increments Δv_1 between the first and second samples should be given the inverse correction or

$$\Delta v_1' = \Delta v_1 \frac{n_0}{n_1} = \Delta v_1 \frac{V_1}{V_1 - s}$$

in which $\Delta v_1'$ is the corrected value. Similarly the withdrawal of the second sample reduces n_1 in the proportion

$$n_2 = n_1 \frac{V_2 - s}{V_2} = n_0 \frac{V_1 - s}{V_1} \cdot \frac{V_2 - s}{V_2}$$

where n_2 is the amount of A remaining. The increment Δv_2 must therefore be corrected by

$$\Delta v_2' = \Delta v_2 \frac{V_1}{V_1 - s} \cdot \frac{V_2}{V_2 - s}$$

and the ith increments should receive corrections by the formula

$$\Delta v_i' = \Delta v_i \prod_{k=1}^{k=i} \frac{V_k}{V_k - s} \tag{1}$$

in which Π represents a progressive product. In table 1 the values of the acid production, given in the second column, have been corrected as just described, with the results given in the third column. (*b*) *The plate counts are to be corrected to the values they would have had if no dilution with titrating fluid took place.* It is evident that withdrawal of samples will have no direct effect upon the plate count. Such counts will, however, be influenced by dilution with the titrating fluid. The plate counts C are therefore corrected to the original *concentration* $3N_0 = n_0/V_0$, of the substance A. When the first sample is taken the concentration, N_1, of A has been reduced to n_0/V_1 so that the counts are corrected by

$$\frac{C_1'}{C_1} = \frac{N_0}{N_1} = \frac{V_1}{V_0}$$

where C_1' is the corrected value. After taking the first sample the amount of A is reduced in the proportion $n_1 = n_0 \cdot (V_1 - s)/V_1$ and its concentration, on taking the second sample, is

$$N_2 = \frac{n_1}{V_2} = \frac{n_0(V_1 - s)}{V_2 V_1}$$

The corresponding correction is therefore

$$\frac{C_2'}{C_2} = \frac{N_0}{N_2} = \frac{V_2 \cdot V_1}{V_0(V_1 - s)}$$

and the correction for the ith plate count is evidently

$$\frac{C'_i}{C_i} = \frac{V_i}{V_0} \prod_{k=1}^{k=i-1} \frac{V_k}{V_k - s} \tag{2}$$

The magnitude of the correction is indicated by comparing the uncorrected and corrected values of the plate count given in columns four and five in table 1.

In the derivations of equations 1 and 2 it has been assumed that the samples withdrawn were all of the same volume, s, as was

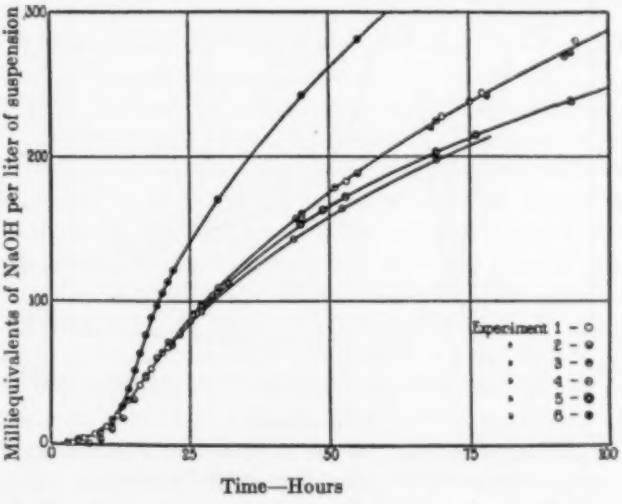

FIG. 3. ACID PRODUCTION OF *L. acidophilus* UNDER VARIOUS CONDITIONS

the case in the experiments. If, however, samples of various volumes are taken the equations will still be correct if in each case the volumes $s_1, s_2 \ldots s_k \ldots s_i$ are progressively substituted for s.

Curves showing the corrected acid production under the conditions outlined for the various experiments listed in table 2 are given in figure 3. It will be observed that the data for experiments 1, 2 and 3, for which the conditions were very closely the same, all lie on a smooth curve, although the measurements were made at intervals that were spread over several months. This

close agreement was, however, apparent only after the curves had been moved a short distance along the axis of abscissae to allow for differences in the inoculation. An objective adjustment of these curves was obtained by making them coincide at the point of maximum rate of production of acid. This point, and its determination, will be discussed later in this article. Allowing for the variations of the original inoculation it will be seen that the experiments attained a high degree of reproducibility.

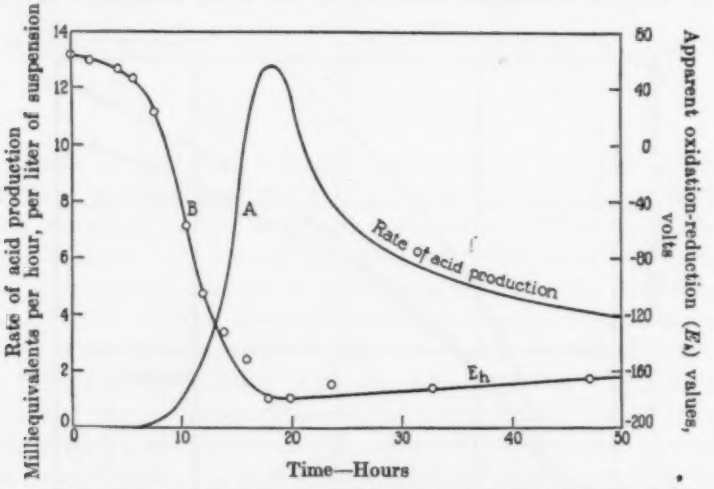

FIG. 4. VARIATION, WITH TIME, OF THE RATE OF ACID PRODUCTION, AND THE APPARENT OXIDATION-REDUCTION POTENTIAL

Although it was found in the previous work that carbon dioxide is entirely necessary for the growth of the organism, no very striking effect was found as a result of increasing the percentage of that component in the saturating gas from 8.1 to 41.6 per cent, the pH being kept at a value of 6.0. The result is seen from the curve for experiment 4 to be a slight decrease of the acid production. An increase of the proportion of oxygen from less than 0.2 to 2.1 per cent also has a comparatively small effect which accords with the description of *Lactobacillus acidophilus* as a facultative anaerobe. As might be expected an increase of the sugar concentration, in this case from 3 to 8 per cent, results in a large increase

in the acid production, as is indicated by the curve plotted of the data for experiment 6.

By differentiation of the curves for acid production, as shown in figure 3, the *rate* of acid production as a function of the time can be obtained. Curve *A* in figure 4 is a typical plot of the rate of acid production (in milliequivalents per liter per hour) as ordinates against the time in hours. The rates were obtained with the aid of a Bausch and Lomb "tangent meter" from a large scale plot of the data for experiment 6 given in table 1. It will be observed that in this case a sharp maximum in the acid production is reached after the growth has proceeded about 18 hours. This is followed by a relatively slow decrease in the rate.

It is of interest to compare curve *A* with a plot, curve *B*, of the trend, with the time, of the apparent oxidation-reduction potentials (E_h values) for the same experiment. The E_h curve shows a slight drop before appreciable amounts of acid are produced, and has an abrupt drop during the time that the rate of acid production is increasing. Furthermore, the maximum in the curve for the rate of acid production occurs at the same time that a minimum in the curve for the E_h is reached. This agreement of maxima and minima in the two types of data is apparently general. It is true of all our experiments, except that for experiment 5 in which there was a high percentage of oxygen in the gas. In that case there was a slight delay in the appearance of the minimum in the E_h curve. It would appear, therefore, that the E_h values are closely related to the rate of metabolism of the organism. A possible explanation is that a reducing substance is produced which is progressively oxidized, or otherwise disposed of, during the growth period. It must be recalled, however, that the E_h as measured is not an equilibrium potential, but is the result of several kinetic processes.[1]

[1] The gas mixtures listed in table 2 were prepared by mixing the commercial tank gases and the analyses were made with the usual Orsat apparatus. The accuracy of the determination of the oxygen present in the mixtures is about ±0.1 per cent, and the figures given for the percentage of oxygen probably represent upper limits. Since the E_h time curves are greatly influenced by small amounts of oxygen we hope to present data based on much more accurate gas analyses, at least for that element, sometime in the future. For comparison it would be very desirable to obtain E_h time curves using mixtures of carbon dioxide

There has been much recent interest in the subject of oxidation-reduction potentials in connection with biochemistry and bacteriology. This is indicated, for instance, by the appearance of three editions in five years of Hewitt's (1931, 1933, 1935) excellent monograph on the subject. Few definite conclusions have been arrived at, largely, it appears to us, because variables have not been controlled. The E_h values are, as is well known, functions of the pH, and corrections for changes of the latter can only be made if more data concerning the composition of the medium are

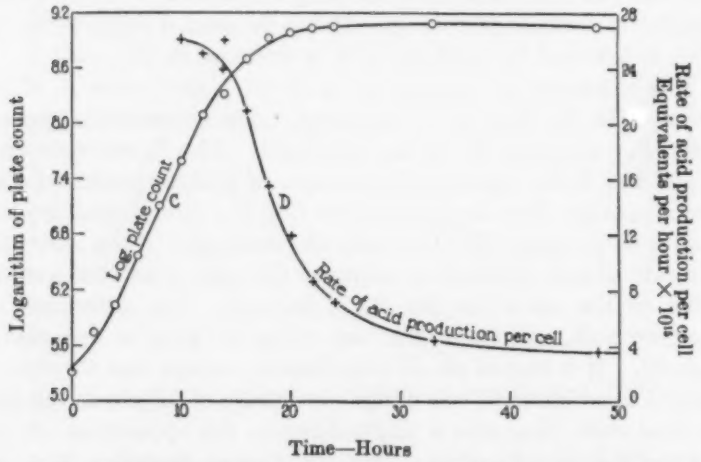

FIG. 5. THE VARIATION, WITH TIME, OF THE PLATE COUNT, AND THE RATE OF ACID PRODUCTION PER CELL

available than is usual in biological studies. It therefore seems probable that interpretations will be facilitated if the pH is kept constant, as it has been in the experiments described in this article. A knowledge of the gas with which the medium is saturated also seems essential, as is a supply of carbon dioxide. The measurements of E_h are to be continued in this Laboratory with a number of improvements in experimental technique which have been suggested by the work described in this article, and further discussion will appear later.

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and nitrogen from which the oxygen has been entirely removed. Unfortunately a method for preparing such mixtures does not, at present, appear to be available.

By combining data on acid production with determinations of the population it is possible to arrive at conclusions concerning the fermenting capacity of single average cells at various portions of the growth period. The results of a typical series of plate counts, corrected as already described, are given, for experiment 6, in figure 5, where curve C passes through the points obtained by plotting the logarithms of the counts against the time in hours. Curve D is the result of dividing the rate of acid production at a given moment, from curve A of figure 4, by the population at the same moment, and plotting the quotient against the time. The ordinates of curve D, which we will call Φ values, therefore represent equivalents of acid per hour, per average unit organism. It will be seen that Φ started with a value of about 26×10^{-15} as soon in the experiment as data were available for its determination. While the population was increasing to its maximum Φ dropped rapidly, and at the end of 50 hours had only 13 per cent of its original value. Our other experiments showed initial values of Φ well within the same order of magnitude as that just given, and the trend with the course of the experiment was about the same. There is, however, evidence that Φ is somewhat greater when the sugar concentration is lower. Rahn (1932) has obtained a value of Φ of 10×10^{-15} for this organism from data taken under widely different conditions from ours. He suggests that his figure may be somewhat low since his population count may have included many dead cells.

Preliminary microscopic studies suggest that the decrease of Φ with time may be, in part, due to decreasing size of the cells. We hope to present quantitative data bearing on this matter in the near future.

By comparing the population curve, C, of figure 5, with curve D of the same figure, and curve A of figure 4 it will be seen that the rate of increase of the number of organisms begins to decrease at about the time that appreciable amounts of acid are formed. Here again the curves given are typical of all our results. It will be recalled that the generation time is inversely proportional to the slope of curve C, and is constant only in the straight line portion ("logarithmic period") of the curve. It would appear therefore that the products of fermentation have the effect of increas-

ing the generation time even though, as in this case, the effect of changing pH is excluded. The generation times for the logarithmic period are given in table 1 for the various experiments. As will be seen they are very roughly constant and are within the limits, 66 to 87, given by Mason (1935).

SUMMARY

The studies previously reported on the growth of *Lactobacillus acidophilus* at constant pH values have been continued. The apparatus which provided automatic pH control has been modified to give (*a*) increased accuracy in determination of the acid production during the early portion of the growth period and (*b*) elimination of the possibility of contamination when the electrodes are inserted into the growth medium. Plate counts have been made and the apparent oxidation-reduction potentials have been followed throughout the growth period. The effects of saturation with various mixtures of nitrogen, oxygen and carbon dioxide, and also a variation of the sugar concentration, have been studied, all at pH = 6.

Slight decreases only in the acid production were observed when the proportions of carbon dioxide and of oxygen were greatly increased.

A correlation was observed between the apparent oxidation-reduction potential and the *rate* of acid production, a maximum in the latter corresponding, in general, with a minimum of the former.

Using the plate counts, the fermenting capacity per unit of time for a single organism could be followed during the growth period. It was found to decrease markedly as the growth proceeded. It was also found that the generation time tends to increase as the products of fermentation accumulate, although the pH is kept constant.

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THE FERMENTATION OF CELLOBIOSE BY BACTERIA[1]

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Jones (1925) and Jones and Wise (1926) found that cellobiose was fermented by *Aerobacter* cultures, but not by members of the *Escherichia* genus, with the exception of one strain of *Escherichia Neapolitana*. Koser (1926) determined the fermentation of cellobiose in a large series of *Escherichia-Aerobacter* cultures isolated from human and animal feces and from soil, and studied its relation to the source of the cultures and to the results of other differential tests. He found that the *Escherichia* cultures obtained from feces failed to attack this carbohydrate, while members of the *Aerobacter* group, whether derived from feces or soil, fermented it readily. The majority of *Escherichia* cultures from soil and practically all of the methyl-red positive, Voges-Proskauer negative and citrate positive *Escherichia-Aerobacter* "intermediate" types, also from soil, fermented it. Later Koser and Saunders (1932) reported that cellobiose was fermented by 4 per cent of the methyl-red positive, Voges-Proskauer negative *Escherichia* cultures from feces. Skinner and Brudnoy (1932) also found that cellobiose was fermented by approximately 10 per cent of the "*B. coli*" strains from human feces. Poe (1934) reported positive fermentation by 20 per cent of the *Escherichia* cultures isolated from water. Finally, we (1935) have reported that 10 per cent of the methyl-red positive, Voges-Proskauer negative *Escherichia* cultures, and all of the *Aerobacter* strains isolated from human feces and urine fermented cellobiose. It

[1] This work was supported in part by contributions from the General Education Board Research Fund and the Department of Biology, College of Arts and Science, University of Rochester.

is evident, therefore, that this carbohydrate is fermented by members of the *Aerobacter* genus and also by a few *Escherichia* cultures. These findings suggest that the fermentation of cellobiose might serve as a convenient additional taxonomic characteristic within the *Escherichia-Aerobacter* group, and possibly in other instances as well. Accordingly, the ability to ferment cellobiose has been determined for over 60 bacterial species, belonging in 18 genera. The results are presented in this report.

METHODS AND MATERIALS

A total of 755 cultures was employed, of which 472 were *Escherichia-Aerobacter* strains isolated recently from human urine and feces and from water. The rest of the cultures were selected from the laboratory collection. The characteristics of most of the cultures were determined in detail to permit exact taxonomic allocation. An exception was made, however, for the sporogenous cultures, in which case the species designations listed are those given by the laboratories from which the cultures were obtained.

The test medium was composed of 0.3 per cent meat extract (Bacto), 0.5 per cent peptone (Bacto), and 0.5 per cent cellobiose (Eastman). It was adjusted to pH 7.2, and then, bromcresol purple was added. The broth was dispensed in Durham fermentation tubes and sterilized for twenty minutes at 15 pounds pressure.

Incubation was at 37°C. for two weeks, unless both acid and gas were produced sooner. The results were recorded daily during the first week, and every second or third day during the second week.

RESULTS

The results are presented in table 1. Approximately one-third of the species fermented this carbohydrate. All *Aerobacter* cultures, regardless of species or source, and almost all of the *Escherichia-Aerobacter* "intermediate" strains decomposed cellobiose producing acid and gas, but none of the typical members of the *Escherichia* group were able to do so. The *Salmonella* cultures differed significantly. *Salmonella aertrycke, Salmonella Schottmülleri*

and *Salmonella enteritidis* produced acid and small amounts of gas, while *Salmonella suipestifer*, *Salmonella paratyphi*, *Salmonella gallinarum*, *Salmonella pullorum* and *Salmonella Morgani* failed to show any evidence of fermentation. A significant difference

TABLE 1

Cellobiose fermentation

ORGANISM	NUMBER OF STRAINS		ORGANISM	NUMBER OF STRAINS	
	Positive	Negative		Positive	Negative
Aerobacter aerogenes	49	0	*Shigella paradysenteriae* (Sonne)	0	18
Aerobacter oxytocum	36	0	*Shigella dispar*	0	11
Aerobacter cloacae	25	0	*Shigella alkalescens*	0	2
Aerobacter levans	2	0	*Shigella ambigua*	0	2
Escherichia-Aerobacter "intermediates"	64	8	*Pseudomonas pyocyaneus*	0	4
Escherichia "coli" group	0	211	*Flavobacterium vitarumen*	1	0
Escherichia "communior" group	0	100	*Chromobacterium violaceum*	0	1
Proteus mirabilis	2	0	*Serratia*, unclassified	5	2
Proteus vulgaris	0	4	*Bacillus cereus*	1	0
Proteus X 19 (Felix)	0	2	*Bacillus subtilis* (Ford)	1	0
Proteus X 19	0	2	*Bacillus subtilis* (Marburg)	1	0
Proteus X 2 (Felix)	0	2	*Bacillus subtilis* (Koch)	0	1
Proteus X Kingsbury (Felix)	2	0	*Bacillus subtilis* (Soule)	0	1
Proteus, unclassified	11	5	*Bacillus anthracis*	0	1
Alcaligenes faecalis	0	3	*Bacillus megatherium*	0	1
Salmonella aertrycke	3	0	*Bacillus mycoides*	0	1
Salmonella Schottmülleri	11	0	*Bacillus niger*	0	1
Salmonella enteritidis	17	2	*Bacillus pseudotetani*	0	1
Salmonella suipestifer	0	6	*Bacillus*, unclassified	5	1
Salmonella paratyphi	0	8	*Staphylococcus aureus*	0	10
Salmonella gallinarum	0	18	*Staphylococcus albus*	1	3
Salmonella pullorum	0	35	*Micrococcus aurantiacus*	1	0
Salmonella Morgani	0	4	*Micrococcus*, unclassified	0	12
Eberthella typhi	15*	0	*Gaffkya tetragena*	0	1
Shigella dysenteriae (Shiga)	0	3	*Neisseria catarrhalis*	0	2
Shigella paradysenteriae (Flexner)	0	8	*Sarcina citrea*	0	1
Shigella paradysenteriae (Hiss and Russell)	0	1	*Sarcina ureae*	0	1
			Rhodococcus rhodochrous	0	2
			Total number of strains	755	

* Delayed fermentation.

was exhibited also between *Eberthella typhi*, which produced acid during the second week of incubation and members of the *Shigella* genus which showed no indication of fermentation. *Proteus mirabilis*, *Proteus* X Kingsbury and eleven of the unclassified *Proteus* cultures fermented cellobiose, usually during the second week of incubation, while *Proteus vulgaris*, *Proteus* X 19 and X 2,

TABLE 2

Correlation of cellobiose fermentation with other differential tests of Escherichia-Aerobacter cultures and with their sources

GROUP	SOURCE AND NUMBER OF CULTURES				TOTAL NUMBER OF CULTURES	METHYL RED TEST	VOGES-PROSKAUER TEST	UTILIZATION OF CITRATE	FERMENTATION OF CELLOBIOSE	FERMENTATION OF α-METHYLGLUCOSIDE	PRODUCTION OF INDOL	PRODUCTION OF HYDROGEN SULPHIDE
	Urine	Feces	Blood or pus	Water or soil								
Aerobacter	21	17	1	56	95	−	+	+	+	+	−	−
	4	6	1	6	17	−	+	+	+	+	+	−
Escherichia	176	108	0	20	304	+	−	−	−	−	+	−
	4	0	0	3	7	+	−	−	−	−	−	−
Escherichia-Aerobacter "intermediates"	0	1	0	1	2	+	−	+	+	+	+	+
	0	5	0	8	13	+	−	+	+	+	−	+
	0	1	0	4	5	+	−	+	+	+	+	−
	0	2	0	0	2	+	−	+	+	+	−	−
	2	4	0	8	14	+	−	+	+	−	−	+
	0	0	1	0	1	+	−	+	+	−	+	−
	1	0	0	0	1	+	−	+	+	−	−	−
	3	1	0	1	5	+	−	+	−	−	+	−
	0	0	1	0	1	+	−	+	−	−	−	+
	1	0	0	0	1	+	−	+	−	−	−	−
	10	5	4	5	24	+	−	−	+	−	+	−
	0	0	1	1	2	+	−	−	+	−	−	−
	0	0	0	1	1	+	−	−	−	−	+	+

and five of the unclassified strains failed to do so. In general, the cocci failed to show any evidence of fermentation. *Micrococcus aurantiacus* and a single strain of *Staphylococcus albus*, however, produced acid promptly.

The relation of cellobiose fermentation to the common differential characteristics of the *Escherichia-Aerobacter* cultures, and to

their sources, is shown in table 2. Typical members of the *Aerobacter* and *Escherichia* genera showed perfect correlation with all of these tests, except for indol production. In the *Escherichia-Aerobacter* "intermediate" group, however, only methyl-red and Voges-Proskauer reactions gave a reciprocal relationship. Of the 72 "intermediate" cultures tested, 64 fermented cellobiose; 45 utilized citrate; 38 produced indol; 31 produced hydrogen sulphide and 22 fermented *a*-methylglucoside. No correlation was found between cellobiose fermentation and the source of the *Escherichia-Aerobacter* cultures; in fact, no reciprocal relationship was observed between any of the differential characteristics and the sources of the strains.

DISCUSSION

The ability to ferment cellobiose is not limited to members of the *Escherichia-Aerobacter* group. Furthermore, the fermentation of cellobiose may be used advantageously for distinguishing certain closely related species or types of bacteria.

For the *Aerobacter* and typical *Escherichia* strains, the fermentation of cellobiose correlates perfectly with the production of acetyl-methyl-carbinol, the methyl-red reaction, the utilization of citrate and the fermentation of *a*-methylglucoside, but not with the production of indol (table 2). For the separation of these two groups, therefore, the fermentation of cellobiose can be employed as well as any other of the reactions. It is better, certainly, than the production of indol. On the other hand, this characteristic alone will not differentiate the *Escherichia-Aerobacter* "intermediate" cultures from both *Aerobacter* and typical *Escherichia* strains. It may be employed, nevertheless, to distinguish species or types within this "intermediate" group. It is significant that 26 cultures, designated tentatively as *Escherichia-Aerobacter* "intermediates," differed from the typical *Escherichia* strains only in their ability to ferment cellobiose. It would seem to us that cultures of this type should be allocated to the *Escherichia* genus.

The results show that neither the fermentation of cellobiose nor any other known characteristic, alone or in combination, will

indicate whether or not the source of the *Escherichia-Aerobacter* strains is fecal or non-fecal. Furthermore, they emphasize the fact that *Aerobacter* and *Escherichia-Aerobacter* "intermediate" strains may be found in human excreta.

The results obtained with the *Salmonella* cultures (table 1) indicate that the fermentation of cellobiose may be employed advantageously for distinguishing certain closely related species. This is especially true in separating *S. aertrycke*, *S. Schottmülleri* and *S. enteritidis* from *S. paratyphi*. It should be emphasized, however, that these cultures must be incubated for at least 10 days to elicit delayed fermentation, although from 4 to 6 days are usually sufficient.

The positive fermentation by the typhoid bacillus, but not by the dysentery bacilli, although significant for the purpose of academic taxonomy, cannot be considered useful in the routine differentiation of these genera, because the former organism is slow and somewhat irregular in its ability to ferment this carbohydrate.

It is evident that *Proteus* cultures differ significantly in their ability to attack cellobiose. This is true, not only for different species, but even for different strains or types. *Proteus mirabilis* fermented cellobiose, while *Proteus vulgaris* did not. Furthermore, of the cultures which had been received directly from Dr. Felix, *Proteus* X Kingsbury attacked cellobiose, whereas the closely related *Proteus* X 19 and X 2 strains failed to do so. These strains of *Proteus* X 19 and X 2 differed, therefore, from those studied by Welch, Mickle and Borman (1934), who reported positive fermentation by all cultures which they tested.

SUMMARY

1. The ability to ferment cellobiose has been determined for 755 bacterial cultures, representing 18 genera and more than 60 species.

2. Approximately one-third of the species fermented this carbohydrate.

3. All of the *Aerobacter* cultures and 90 per cent of the *Escherichia-Aerobacter* "intermediate" strains produced acid and gas,

while none of the typical *Escherichia* showed evidence of fermentation.

4. The correlation of cellobiose fermentation with other characteristics of the *Escherichia-Aerobacter* cultures, and with their sources, is recorded.

5. The fermentation of cellobiose will differentiate *Aerobacter* from typical *Escherichia* strains, and can be employed advantageously for distinguishing species or types within the *Escherichia-Aerobacter* "intermediate" group. On the contrary, it will not indicate whether the source of *Escherichia-Aerobacter* cultures is fecal or non-fecal.

6. The value of cellobiose fermentation as an additional aid in the separation of certain closely related *Salmonella* and *Proteus* cultures is indicated.

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FERMENTATIVE VARIABILITY OF SHIGELLA PARADYSENTERIAE, SONNE

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The most important cultural characteristic by which the Sonne type of the dysentery bacillus is differentiated from other members of the paradysentery group is its late fermentation of lactose. As is well known this action upon lactose is due, not to the slow utilization of the sugar by the original organism but to the invariable appearance in media containing lactose of a variant which attacks the sugar vigorously. In tube cultures of the organism this variant usually makes itself evident after several days' incubation, hence the reaction is often spoken of as slow lactose fermentation. In a recent article by Sears, Bilderback, Ashley and Rohner (1935) it was pointed out that a similar behavior in sucrose media is shown by the Sonne organism and that the two variations are entirely independent of each other. The reactions manifest themselves by late indicator change in liquid media and by the appearance after several days' incubation of secondary colonies on solid media containing the sugar.

In the above cited paper it was also pointed out that the strains of the organism investigated gave a late fermentation of raffinose upon liquid media but that secondary colonies upon solid media containing this trisaccharide were not obtained. The purpose of the present paper is to report the procuring of a stable variant of the Sonne dysentery bacillus which ferments raffinose vigorously in twenty-four hours or less and to show that this variation is independent of the variation of the organism with respect to its fermentation of lactose and sucrose.

The strains used were all typical morphologically and culturally and gave good agglutination with a Sonne serum. Plated

on raffinose agar, containing Andrade's indicator, they gave in five to seven days secondary colonies appearing as small pinkish or red papillae. When these were picked off and replated on raffinose litmus agar some colonies were quite red in twenty-four hours. Replating of these red colonies usually produced only red colonies. The latter failed to form secondary colonies when incubated as long as a month, and when inoculated to broth tubes containing raffinose and Andrade's indicator gave a marked red color in eighteen to twenty-four hours. This raffinose variant could be obtained from the parent organism, the lactose variant, the sucrose variant or the variant which fermented both the latter sugars. Its vigorous fermentation of lactose and sucrose was in no way affected by its new power to attack raffinose.

With one strain of the Sonne bacillus, marked "Hodgson" in our collection, we have obtained seven separate variants differing from each other with respect to the fermentation of the three sugars, lactose, sucrose and raffinose. In other respects these cultures seem to be identical with the parent culture. They all agglutinate to about the same titer with a serum prepared against the parent culture and their utilization of other fermentable substances remains the same. Likewise the S $\rightarrow$ R variation, which often occurs spontaneously in a Sonne culture, seems to take place readily with each of the variants. The rough variant is also able to undergo variation with respect to its fermentation of these sugars, although remaining rough in the process. For example when selecting a colony for isolation of the sucrose variant a rough colony was picked. The culture procured gave a large dryish colony with uneven edges which agglutinated spontaneously when suspended in salt solution. When this rough culture was grown on raffinose a rapid raffinose-fermenting culture was obtained which also had the above rough characteristics. Nungester (1933) has reported a strain of *Escherichia coli* from which he was able, in much the same manner as we have done, to obtain seven variants showing different fermentation reactions on lactose, maltose and sucrose.

Table 1 gives the principal differential characters of the parent and seven variant cultures of our organism.

It has been our experience in the study of many Sonne strains and also of many *mutabile* strains of *Escherichia coli* and other organisms that the time of appearance of an acid reaction in broth media containing a sugar upon which the organism will produce a variant will vary greatly even with the same strain. In fact we have observed that if, from the same plate, cultures are made from a large number of colonies the time of appearance of a marked acid reaction may vary from thirty-six hours to as long

TABLE 1

Chief differential characters of the Hodgson strain of S. paradysenteriae, Sonne and its seven variants

CULTURE	LACTOSE BROTH	SUCROSE BROTH	RAFFINOSE BROTH	SECONDARY COLONIES ON AGAR CONTAINING			HIGHEST DILUTION IN WHICH A SERUM PREPARED AGAINST THE PARENT STRAIN AGGLUTINATES
				Lactose	Sucrose	Raffinose	
1. Parent culture	A (late)	A (late)	A (late)	+	+	+	1:3200
2. Lactose variant	A	A (late)	A (late)	−	+	+	1:3200
3. Sucrose variant	A (late)	A	A (late)	+	−	+	1:3200
4. Raffinose variant	A (late)	A (late)	A	+	+	−	1:3200
5. Lactose-sucrose variant	A	A	A (late)	−	−	+	1:3200
6. Lactose-raffinose variant	A	A (late)	A	−	+	−	1:3200
7. Sucrose-raffinose variant	A (late)	A	A	+	−	−	1:3200
8. Lactose-sucrose-raffinose variant	A	A	A	−	−	−	1:3200

A indicates definite and early indicator change, appearing in all cases in less than twenty-four hours and sometimes as early as six hours after inoculation.

A (late) indicates that indicator change occurred only after two or more days (see text).

as three weeks or more. The time it takes to produce secondary colonies on solid media is somewhat more regular, though variations are met with here also.

Some authors have reported that their Sonne strains gave delayed fermentation of maltose. We have studied about twenty-five strains isolated locally in the last two years and all but one have given prompt fermentation of this sugar. This one fermented in less than forty-eight hours but gave secondary colonies on maltose agar. We did not isolate the pure variant but we

have no doubt it could have been done. Welch and Mickle (1932) stated that some of their strains gave delayed fermentation of the four sugars, lactose, sucrose, maltose and raffinose. With such a strain it should be possible to develop fifteen variants.

SUMMARY

A strain of *Shigella paradysenteriae*, Sonne which gives delayed fermentation of the three sugars, lactose, sucrose and raffinose is capable, under suitable conditions, of giving rise to seven variant strains which differ from each other in their capacity to ferment these substances. Each of the variants is identical with the parent organism in its specific agglutinability and in its other biochemical characters. The differential characters of the variant strains remain unchanged when S → R dissociation is carried out.

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THE CLASSIFICATION OF A GROUP OF ESCHERICHIA ISOLATED FROM THE INTESTINAL TRACT OF PATIENTS WITH ULCERATIVE COLITIS

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During the past year an investigation was carried out concerning the incidence and biological characteristics of the hemolytic *Escherichia* (*Bacillus coli*) in the intestinal tract of patients with chronic ulcerative colitis (Nicholls, 1935). The specimens were obtained from patients in the gastrointestinal clinic of the New York Hospital, through the courtesy of Dr. Thomas T. Mackie. One hundred and fifty-five strains, 66 hemolytic and 89 non-hemolytic, were isolated from 106 cultures. In this study only the usual biological tests for the identification of the organisms as belonging to the genus *Escherichia* were carried out and the strains were divided into two groups, the *Escherichia coli* (*communis*) and the *Escherichia communior*, according to their reaction to sucrose, as originally outlined by Smith (1895).

The present investigation is a further study of these strains to determine the species to which each of them belongs and to see if any one species predominates. The nomenclature of Bergey (1930) is used throughout the paper.

BIOLOGICAL CHARACTERISTICS OF THE STRAINS OF ESCHERICHIA THAT WERE ISOLATED

Various tests were carried out on the strains of *Escherichia*, hemolytic and non-hemolytic, for their identification. These tests were performed as soon after isolation of the strains as possible. All of the organisms grew well in litmus milk with the production of acid and clot. On Russell's medium they gave the

characteristic appearance, with acid and gas in the butt and acid in the slant. All of the strains produced indol in peptone solution. None of the strains liquefied gelatin at 22°C. in 21 days. The Voges-Proskauer and methyl-red tests were carried out on all of the strains. The mediums were prepared according to the method described by Clark and Lubs (1915), and the technic followed was that outlined by Levine (1916). All of the strains were Voges-Proskauer negative and methyl-red positive.

Sugar fermentations. The mediums were prepared and the tests carried out in the following manner:

One per cent peptone and 0.5 per cent sodium chloride were dissolved in a 1.5 per cent agar solution. To this mixture was added 1 per cent of the desired sugar, with the exception of maltose. For an indicator, 1 per cent Andrade's solution was used. The medium was tubed in 5 cc. portions and sterilized in the Arnold for 20 minutes, on three successive days, with the exception of inulin which was autoclaved.

As maltose is hydrolyzed by heat it was made up in a 20 per cent solution and filtered through an L_5 Pasteur-Chamberland filter. This filtrate was added, under sterile precautions to the sterilized agar-peptone-salt medium, while warm, in amounts to make a 1 per cent solution, and 5 cc. portions of the sugar medium were immediately put into sterilized test tubes, cooled and incubated for 24 hours to test for sterility.

The tubes were heated in a water bath until the agar medium was melted, and then cooled to about 47°C. To the contents of each tube was added 0.2 cc. of a 24-hour broth culture of the organisms; the mixture was shaken and allowed to cool. A minimum of 8 days' incubation was allowed before the final reading was made and the tube was discarded. A result was considered positive for acid when a pink color appeared. The presence of gas was determined by a breaking and cracking of the medium. For the adonitol determinations, the agar was omitted and Dunham fermentation tubes containing 4 cc. of the sugar medium were used. After inoculating and incubating the medium, a bubble in the small inverted tube showed the presence of gas, and a pink color, the presence of acid.

All of the strains examined fermented lactose, maltose, glucose and mannitol with the production of acid and gas. Twenty-eight of the hemolytic strains and 30 of the non-hemolytic strains formed acid and gas in sucrose. There was great variation in

the fermentation tests with salicin, dulcitol, and adonitol. Forty-three of the hemolytic strains and 52 of the non-hemolytic strains fermented salicin, while 35 of the hemolytic and 53 of the non-hemolytic strains reacted with dulcitol. Only 11 of the hemolytic and 11 of the non-hemolytic strains formed acid and gas in adonitol. One strain formed acid but no gas in salicin.

The sucrose and salicin fermentation tests were carried out in both solid (agar) and liquid mediums. Twelve strains which failed to produce acid and gas with sucrose in the solid medium gave a positive result in the liquid medium. Also with salicin, 23 strains which failed to react in the solid medium produced acid and gas in the liquid medium. After subculturing the strains several times and allowing the inoculated sugar cultures to incubate from 7 to 14 days, all of the sucrose cultures and all but 2 of the salicin cultures gave a positive reaction in the solid mediums.

A careful study was made of the motility of the organisms. The strains were subcultured daily and grown in plain broth and blood broth, and on agar medium. A strain was not considered definitely non-motile until it had been examined at least 5 times. For all of the organisms that failed to show motility, a Casares-Gil stain (Zinsser and Bayne-Jones, 1934) was made and in no instance was the presence of flagella demonstrated. Motile organisms were used as controls. Thirty-seven of the hemolytic strains were motile and 48 of the non-hemolytic.

It was found that the 155 strains in the group differed from each other only in their reactions to sucrose, salicin, dulcitol, and adonitol, and in the presence or absence of motility. All other biological characteristics were shared in common. The strains may be classified, therefore, as various species of the genus *Escherichia*.

Having determined that the organisms recovered from the intestinal tract were members of one large group it was thought advisable to study them separately and group them according to their individual reactions. It was found that the organisms belonged to 21 different groups, with from 1 to 23 strains in each. In table 1 are shown the 21 groups, with the reactions of each to

sucrose, salicin, dulcitol, and adonitol, and the presence or absence of motility. The number of strains falling into each group is also indicated, and their division into hemolytic and non-hemo-

TABLE 1

Classification of the strains of Escherichia according to their reactions

GROUP	SUCROSE	SALICIN	DULCITOL	ADONITOL	MOTILITY	TOTAL NUMBER OF STRAINS	NUMBER OF HEMOLYTIC	NUMBER OF NON-HEMOLYTIC	SPECIES
1	−	−	−	−	−	19	6	13	*Esch. vesiculiformans* (Henrici)
2	−	−	−	−	+	5	3	2	*Esch. paragrünthali* (Castellani)
3	−	−	−	+	−	1	0	1	*Esch. acidilactici* (Hüppe)
4	−	−	−	+	+	2	1	1	
5	−	−	+	−	−	5	1	4	
6	−	−	+	−	+	12	4	8	*Esch. metacoloides* (Castellani)
7	−	+	−	−	−	10	3	7	*Esch. colitropicales* (Castellani)
8	−	+	−	+	−	6	3	3	
9	−	+	−	+	+	6	4	2	
10	−	+	+	−	−	11	5	6	*Esch. Schaefferi* (Castellani and Chalmers)
11	−	+	+	−	+	19	8	11	*Esch. coli* (Escherich)
12	−	A	+	−	+	1	0	1	*Esch. cavicida* (Brieger)
13	+	−	−	−	−	5	3	2	*Esch. pseudocoscorobae* (Castellani and Chalmers)
14	+	+	−	−	−	5	4	1	
15	+	+	−	+	−	3	1	2	
16	+	+	−	−	+	3	2	1	
17	+	+	−	+	+	2	1	1	*Esch. pseudocoloides* (Castellani) (Bergey)
18	+	+	+	−	−	5	3	2	*Esch. Neapolitana* (Emmerich)
19	+	+	+	−	+	23	8	15	*Esch. communior* (Durham)
20	+	−	+	−	+	10	5	5	*Esch. communior* (Kligler)
21	+	+	+	+	+	2	1	1	*Esch. metacoli* (Castellani)

+, production of acid and gas, or presence of motility; −, a negative result; A, acid without gas.

lytic types. Where the reactions of a group conform to those of a known species, the name of that species is included in the table.

Of the 21 groups, the members of 14 had fermentative powers similar to known species, while in those of 7 groups the reactions were variable. One hundred and twenty-five of the 155 strains belonged to the 14 groups of known species, leaving only 30

strains as members of the 7 unnamed groups. The largest number of strains (23) belonged to the species *Escherichia communior*, while only 19 were *Escherichia coli* (*communis*). Other species which were well represented were *Escherichia vesiculiformans* with 19 strains, *Escherichia metacoloides* with 12, *Escherichia colitropicalis* with 10, *Escherichia Schaefferi* (Castellani and Chalmers) with 11, and *Escherichia communior* (Kligler) with 10. All of the other species had 6 or less strains each.

From many of the patients repeat cultures were obtained from which the hemolytic and non-hemolytic *Escherichia* were isolated. It was a matter of interest to determine whether all of the strains from one person were of the same species, and it was found that this was not the case. The following is a representative series from patient 86556:

DATE	HEMOLYTIC	NON-HEMOLYTIC
1935		
January 21	*Esch. paragrünthali*	*Esch. Schaefferi*
February 8	*Esch. Neapolitana*	*Esch. vesiculiformans*
February 26	*Esch. Schaefferi*	
March 8	*Esch. communior*	*Esch. acidilactici*
March 29	*Esch. Neapolitana*	Group 4

DISCUSSION

Escherichia communior is believed to be the most common species of the genus *Escherichia*. This was found to be true in the present study, as 23 strains, the largest number falling into any individual group, belonged to that species. Ten strains gave reactions which differed from the *Escherichia communior* only in their failure to ferment salicin. Kligler (1914) encountered a group of strains with characteristics similar to these, and he classified them as belonging to the species *Escherichia communior*. Castellani and Chalmers (1919) and Bergey (1930) describe *Escherichia communior* as salicin-positive, and neither investigator mentions a species with reactions similar to the strains of Kligler and those isolated in this investigation.

Escherichia vesiculiformans is not one of the species more commonly found in the intestinal tract, but in the present study there

were 19 strains of this species. Two other unusual species that had more than 10 strains were *Escherichia metacoloides* and *Escherichia colitropicalis*. One strain only conformed to the reactions of *Escherichia acidilactici*, although this is considered a common inhabitant of the intestinal tract. This species is probably confused with *Escherichia vesiculiformans* because their reactions are similar except in the case of adonitol.

Escherichia Schaefferi is claimed by Castellani and Chalmers (1919) to be nonmotile and to produce acid and clot in litmus milk, while Bergey (1930) states that it is motile and produces acid only in milk. Eleven strains had reactions similar to those outlined by Castellani and Chalmers while none were encountered like those described by Bergey.

There were 7 groups consisting of from 2 to 6 strains each, the reactions of which did not conform to any of the species reported in the literature. These may be species of the genus *Escherichia* which occur in such small numbers that they have not been previously classified, or they may be variants of closely related known species, as in nearly every case each of the unnamed species differs from a known species in one reaction only. However, the differentiation among the known species themselves is, in many instances, based solely on a change in one reaction.

The strains isolated from several cultures obtained from the same individual were found to belong to various species. Also, where a hemolytic and a nonhemolytic strain were isolated from the same specimen they usually belonged to different species. From this it may be concluded that several species of the genus *Escherichia* are present in the intestinal tract at the same time. Moreover, the different species may be so closely related that their biological characteristics are not fixed but change from time to time in the intestinal tract.

Sixty-six of the 155 strains showed the presence of hemolysis on blood agar plates. These strains were distributed through the 21 different groups and in most instances represented from one-third to one-half of the members of each group.

CONCLUSIONS

1. One hundred and fifty-five strains of the genus *Escherichia* were isolated from 106 samples of feces obtained from the intestinal tract of patients with chronic ulcerative colitis. Sixty-six of the strains were hemolytic and 89 non-hemolytic. The biological characteristics of the organisms were studied to determine their species.

2. All strains having similar reactions were allotted to one group, and 21 different groups were represented. The members of 14 of the groups which included 125 strains had fermentative powers and motility or non-motility similar to known species. Seven groups including 30 strains had reactions which did not conform to any of the species described in the literature.

3. *Escherichia communior* was the species best represented with 23 strains. *Escherichia coli* and *Escherichia vesiculiformans* had 19 strains each. Other species which were well represented were *Escherichia metacoloides* with 12, *Escherichia colitropicalis* with 10. *Escherichia Schaefferi* with 11, and *Escherichia communior* (Kligler) with 10. All of the other species had 6 or less strains each.

4. The presence of hemolysis was not a significant factor in the classification of the strains.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

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BACTERIOLOGY AND THE PUBLIC HEALTH. *Fred O. Tonney*, Director, Technical Service and Research, Board of Health, Chicago, Ill.

BACTERIOLOGY AND THE MEAT PACKING INDUSTRY. *Lloyd B. Jensen*, Bacteriologist, Swift and Co., Chicago, Ill.

PLANT NUTRITION AND DISEASE RESISTANCE. (Illustrated.) *George K. K. Link*, University of Chicago, Chicago, Ill.

THE AGGLUTINATION REACTION IN THE CLASSIFICATION OF SPORE-FORMING ANAEROBES. *L. S. McClung*, Research Division, American Can Company, Maywood, Ill.

The value of the agglutination reaction in the classification of the spore-forming anaerobes was considered in a review of the literature including both the older papers and those appearing in which use is made of the H—O technique (receptor analysis hypothesis). The new technique was shown to be of definite advantage in determining the exact antigenic constitution of certain species studied. Stress was given to the study of large numbers of strains of a given species, where available, and to carefully executed absorption tests. The existence of strains deficient in the heat labile fraction was recognized and the suggestion made that the studies of unknown species include the possibility of such strains. The species considered in detail were *Clostridium tetani*, *Cl. Chauvoei*, *Cl. acetobutylicum*, *Cl. felsineum*, *Cl. roseum*, *Cl. thermosaccharolyticum*, *Cl. bifermentans*, *Cl. centrosporogenes*, *Cl. botulinum*, *Cl. parabotulinum*, *Cl. sporogenes*, *Cl. parasporogenes*, and *Cl. Welchii*, and the *vibrion septique*.

STUDIES OF STREPTOCOCCI

I. QUALITATIVE DIFFERENCES IN RESISTANCE TO VARIOUS AGENTS[1]

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The differentiation of "pathogenic" from "non-pathogenic" types of those streptococci that do not produce exotoxin presents a difficult problem. This series of studies was undertaken in the hope of finding a satisfactory method.

The strains used were isolated from suspected foci of infection (pharynx, tonsils, teeth, gum margins, feces, and other foci) of patients with chronic diseases (arthritis, upper respiratory tract infection, certain eye diseases, "intestinal intoxication," "colitis," etc.). Differences in resistance of these strains to certain injurious substances will be described in the present paper.

Because of the difficulty of obtaining uniformly dispersed suspensions of rough strains, these preliminary studies were confined to smooth strains.

Because anomalous results were obtained with enterococci (*Streptococcus fecalis*), the study of this organism will receive treatment in a separate paper.

THE SELECTIVE ISOLATION OF SMOOTH SUB-STRAINS[3] FROM MIXED CULTURES

The method used for isolating smooth strains of streptococci was described in detail elsewhere (Rawls and Chapman, 1935) and consisted of the following steps:

[1] Aided by grants from the Ophthalmological Foundation, Inc.

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[3] The purified variants are referred to as "sub-strains" because several variants may represent the same parent strain and, when separated, they become individual "strains" in the generally accepted use of the word.

Swabs of suspected foci were placed in recently boiled and cooled tall tubes of brain heart infusion and incubated overnight. The following morning, the swabs were withdrawn and the tubes gently rotated. They were then allowed to stand in the incubator for a few hours to permit settling of the coarser particles. Transplants were made from just below the surface and plated on blood agar. The plates were spread by means of glass "spreaders." Because anaerobic cultivation on blood agar sometimes revealed differences which were not apparent by aerobic methods, the plates were incubated overnight in Varney type anaerobic jars. The following morning, the growths were critically examined by transmitted and reflected light. With certain strains, the colonies were so minute or their hemolytic zones on blood agar were so faint that recognition of these colonies required careful searching of the plates.

Based on any minute differences in appearance, each type of colony was transplanted to another blood agar plate for purification. After overnight incubation in anaerobic jars, the plates were inspected for hemolysis and purity, and impure cultures were further purified. The pure cultures were harvested by means of metal scrapers and transferred to bottles of brain heart infusion. After overnight incubation, each growth was inspected for smoothness and tested for its bile solubility and staining reactions. Transplants were made to confirm purity and hemolytic properties.

There is a distinct tendency for certain strains to degenerate to the SR and R types. To minimize degeneration, an effort was made to obtain pure cultures as soon as possible after isolation. Strains which gave off variants continually and could not be completely separated were discarded.

CORRELATION BETWEEN THE RESISTANCE OF STREPTOCOCCI TO THE "BACTERICIDAL" ACTION OF FRESH BLOOD AND TO SODIUM CARBONATE AND BICARBONATE

Purified sub-strains having been obtained, certain *in vitro* tests were applied. One of the earliest used was the "bactericidin" reaction which had been used by other investigators in the study

of streptococci and other organisms. It was shown elsewhere (Rawls and Chapman, 1935) that there was a correlation between the resistance of streptococci to the "bactericidal" action of diluted, fresh, defibrinated guinea pig blood and their tendency to produce arthritis in rabbits. During the course of the experiments, it had been noted that: (1) rabbits injected with strains that were *resistant* to the "bactericidal" agent frequently developed toxic symptoms after the injection of small amounts of culture; and (2) most rabbits inoculated with strains that were *sensitive* to the "bactericidal" agent could be given much larger amounts before toxic symptoms occurred. The possibility that the "bactericidin" reaction might be correlated with toxicity of streptococci prompted further study of the reaction.

The technic employed for the "bactericidin" test was as follows: Blood was aseptically obtained by heart puncture of a number of guinea pigs and defibrinated with glass beads. It was then pooled and divided into two portions, one of which was heated to 52° for 30 minutes to inactivate complement. Higher temperatures, such as are used to inactivate hemolytic complement in serum, frequently caused hemolysis of the whole blood and a tendency to clot. Portions of the heated and unheated blood were separately diluted with 3 volumes of sterile saline. Dilution with nutritive media, instead of with saline, confused the readings. Sterile plugged tubes were placed in a rack containing two rows of holes. To each tube in the front row was added 0.5 cc. of diluted *unheated* blood. The tubes in the rear row, which received 0.5 cc. of the diluted *heated* blood, served as controls. A loopful of an overnight brain heart infusion culture of each sub-strain was added to each of a pair of tubes, one of which contained unheated blood while the other contained heated blood. After incubation for 48 hours, a loopful of each blood-bacteria mixture was transplanted to a blood agar plate, making a streak 2 cm. long. After overnight incubation, the growths from the streaks were compared.

If the growth from a fresh (unheated) blood mixture was similar to that from the corresponding heated blood control, indicating considerable resistance to the "bactericidal" agent, the reaction was recorded as ++++. If the growth from the fresh blood

was slightly diminished as compared with that of the control, the reaction was recorded as +++. A poor growth from fresh blood, contrasted with a good growth from the control was recorded as ++ or +. The absence of growth from fresh blood, compared with a good growth from the control indicated maximum sensitivity to the "bactericidal" agent and was considered a negative reaction.

This "bactericidin" reaction, when applied to a large number of sub-strains of streptococci, gave reproducible results. Further, in a series of experiments to be reported later, there appeared to be a correlation between resistance of a sub-strain as measured by this test and its ability to kill rabbits when injected intravenously in live culture. However, because it required 3 days to complete the test, and because the potency of guinea pig blood might vary from time to time, other *in vitro* tests were sought.

It was recalled that, in attempting to isolate arthrotropic streptococci from feces, Burbank (1929) described a method in which sodium carbonate was used to eliminate the growth of colon-group organisms. This method was used by Hadjopoulos and Burbank (1930), Stainsby and Nichols (1932) and Benson (1932, 1934). The principle was also employed by Groesbeck (1935) for the isolation of streptococci from milk and by Mueller and Whitman (1931) for the isolation of hemolytic streptococci from throat swabs.

In considering the possible mechanism of the method, it was reasoned that the survival of streptococci after treatment with sodium carbonate solution might be related to the factor responsible for the survival of certain strains after treatment with fresh blood. To test this theory, the resistance of streptococci to the "bactericidin" of fresh blood was compared with their resistance to sodium carbonate, the latter being determined as follows:

To a number of tubes were added 0.5 cc. quantities of sterile 1.0 per cent sodium carbonate (Merck reagent). Each tube was then inoculated with a loopful of an 18-hour brain heart infusion culture of a purified sub-strain. A loopful from each tube was transplanted immediately to a blood agar plate to serve as a control. The tubes were put in the incubator. At the end of 1 hour,

a second loopful from each tube was transplanted to blood agar and incubated overnight. The following morning, the growths from the two sets of transplants were compared.

The readings were interpreted in a manner similar to that used for the "bactericidin" reaction. Identical growths from the initial (control) and final transplants were considered ++++ reactions, while a diminished growth from the final transplant when compared with that from the initial transplant was recorded as +++, ++ or negative according to the relative reduction of growth.

In some instances there was evidence of excessive injury to the growths. In order to reduce the action of the 1.0 per cent sodium carbonate, the concentration was reduced to 0.5 per cent and 132 strains were tested by exposing them to this solution for exactly 1 hour.

Agreement with the "bactericidin" test (difference of 0 or +) was obtained in 70.4 per cent; partial agreement (difference of ++) was noted in 18.2 per cent; and disagreement (difference of +++ or ++++) occurred in 11.4 per cent of the strains. However, most of the growths were weaker than corresponding ones obtained with the "bactericidin" test. This was attributed to the strongly alkaline reaction of the sodium carbonate solution. Further tests were made with sodium bicarbonate.

When 214 strains were tested with 0.25 per cent sodium bicarbonate and the results compared with the "bactericidin" reactions of the strains, there was agreement between the two tests in 91.4 per cent. With a concentration of 0.3 per cent sodium bicarbonate, agreement with the "bactericidin" test occurred in 96.6 per cent of 683 strains.

TESTS WITH OTHER CHEMICALS

The possibility was then considered that other substances might be used in place of fresh blood, sodium carbonate and sodium bicarbonate. It was reasoned that, since these chemically different substances gave similar results, the resistance of a strain to these agents was possibly due to the possession by the strain of a non-specific resistance factor and not to a specific action of

the reagent used. If this were true, it should be possible to obtain similar results with other substances.

Obviously, if the test solution should be too concentrated, all the strains will fail to grow but, with progressive dilution, increasingly, more strains should survive. It was postulated that certain intermediate dilutions should permit growth of only the more resistant strains and, within this range, there should be a critical dilution which should give results correlating with the "bactericidin" test.

Preliminary tests of this theory were made with 1:100,000 "Merthiolate"[4] and 1:1000 phenol (Merck reagent). The results were similar to those obtained with the "bactericidin" reaction. This indicated that, with appropriate dilution of any injurious substance, it should be possible to obtain a correlation with the "bactericidin" reaction.

To study this phenomenon more completely, a number of strains were selected for comparison with a more extensive series of chemicals, using the "bactericidin" reaction for reference. Because of the large number of tests which would be necessary for the titration of each chemical with a considerable number of strains, preliminary tests were made with concentrations which were arbitrarily chosen on the basis of estimated relative antibacterial properties of the chemical substances.

The procedure used was similar to that employed in the previous chemical tests. The following results were obtained: With 0.3 per cent sodium bicarbonate (Merck reagent), 0.2 per cent sodium carbonate (Merck reagent), 1:5000 phenol (Merck reagent), 1:10,000 basic fuchsin (National Aniline, certified) and 1:100,000 hexylresorcinol ("S.T. 37," diluted 1:100) there was a good correlation with the "bactericidin" reaction (table 1). However, poor growths were obtained after exposure of the strains to 1:10,000 crystal violet (National Aniline, certified), 1:5000 mercuric chloride (Eimer & Amend, T. P.), 1:500,000 "Merthiolate" (Lilly) and 1:10,000 mercurochrome (H. W. & D.) due to

[4] The word "Merthiolate" used in this and the following papers refers to sodium ethyl-mercuri-thiosalicylate, sold by Eli Lilly and Company under the trade mark "Merthiolate."

TABLE 1

Resistance of streptococci to different agents

STRAIN NUMBER	ORGANISM	"BACTERICIDIN" REACTION	CHEMICAL CONTROL	1 HOUR'S EXPOSURE TO					
				$NaHCO_3$, 0.20 per cent	Na_2CO_3, 0.20 per cent	Phenol, 1:5000	Basic fuchsin, 1:10,000	Hexylresorcinol, 1:100,000	"Merthiolate," 1:500,000
7379	Streptococcus, alpha	++++	++++	++++	++++	++++	++++	++++	+
7372	Streptococcus, alpha	+++	++++	++++	+++	+++	+++	++++	++++
7387	Streptococcus, alpha	++++	++++	+++	++	+++	++	+++	0
7388	Streptococcus, alpha	++++	++++	++++	++	+++	+++	+++	+
7380	Streptococcus, alpha	+++	++++	++++	+++	0	+++	++++	0
7378	Streptococcus, alpha	++	++++	++++	++++	++	++	++	0
7376	Streptococcus, alpha	+++	++++	0	0	++	++	++	++
7366	Streptococcus, alpha	−	++++	0	0	+	0	+	+
7362	Streptococcus, gamma	++++	++++	++++	++++	++++	++++	++++	0
7386	Streptococcus, gamma	+++	++++	++++	+	+++	+++	+++	0
7368	Streptococcus, gamma	−	++++	++	++	0	0	++	0
7365	Streptococcus, gamma	++	++++	0	0	0	+	+	+
7371	Streptococcus, gamma	++	++++	0	0	0	0	0	0
7377	Streptococcus, gamma	++	Few*	0	0	0	0	0	0
7382	Streptococcus, gamma	++	Few	0	0	0	0	0	0

* Only a few colonies developed.

an underestimate of the intensity of the antibacterial properties of these solutions.

In a more extensive series of tests with hexylresorcinol, it was found that a still better correlation with the "bactericidin" test (96.6 per cent, on the basis of 439 strains) could be obtained by reducing the concentration of hexylresorcinol to 1:200,000. When tests with the 1:200,000 solution and with 0.3 per cent sodium bicarbonate were applied to 268 strains, there was an agreement between these two tests in 97.8 per cent of the strains. In a later series of 1868 strains, the agreement was 99.2 per cent.

Certain strains grew poorly even from control tests made with distilled water. In attempting to obtain better growths, tests were made by dissolving the hexylresorcinol and sodium bicarbonate in brain heart infusion instead of in water but the results were no different. In fact, control tests made with brain heart infusion alone also gave similar results. Therefore, any of the solutions could be used as a control.

The above results suggest that the types of streptococci studied possessed varying amounts of non-specific resistance factor which could be determined by exposure to appropriate time/dilutions of a number of different chemical substances as well as to the "bactericidal" action of diluted, fresh, defibrinated guinea pig blood. These results agree with the conclusion of Thompson and Megrail (1934) that there is a correlation between sensitivity of streptococci to various agents.

SUMMARY

Smooth strains of streptococci, which had been isolated from suspected foci of infection of patients suffering from chronic diseases, varied in their resistance to the "bactericidal" action of diluted, fresh, defibrinated guinea pig blood.

Resistance to this "bactericidal" agent was correlated with resistance to appropriate time/dilutions of sodium carbonate, sodium bicarbonate, hexylresorcinol, phenol, basic fuchsin, "Merthiolate," etc.

A series of 1868 strains was tested by exposing them to 1:200,000 hexylresorcinol and 0.3 per cent sodium bicarbonate

for 1 hour and comparing the reduction of viable organisms by means of blood agar transplants. Similar results were obtained with both solutions in 99.2 per cent of the strains.

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STUDIES OF STREPTOCOCCI

II. QUANTITATIVE DIFFERENCES IN RESISTANCE TO SODIUM BICARBONATE AND HEXYLRESORCINOL[1]

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It was shown in the previous paper (Chapman and Rawls, 1936) that different streptococci vary in their resistance to injurious agents but that a particular "sub-strain" shows similar resistance to a number of injurious agents.

The "sub-strains" used were purified smooth variants of streptococci (but not enterococci) that were isolated from suspected foci of infection of patients with chronic diseases.

When 1868 sub-strains were tested by exposing them for 1 hour to 0.3 per cent sodium bicarbonate and to 1:200,000 hexylresorcinol and testing for viability, 99.2 per cent of the cultures gave identical results in both tests. Therefore, it was postulated that resistance to an injurious agent was dependent upon the elaboration of a non-specific factor by the organism and was not related to any specific action of the injurious agent. This suggested that, with appropriate time/dilutions of injurious agents, the resistance of a culture to these injurious agents depends on the quantity of non-specific resistance factor elaborated by the culture.

In the present paper we shall try to show that this resistance factor can be measured quantitatively by an extension of these methods.

Because many of the strains exhibited maximum resistance to the bicarbonate and hexylresorcinol solutions, attempts were made to measure higher degrees of resistance by including addi-

[1] Aided by grants from the Ophthalmological Foundation, Inc.

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tional tests with stronger solutions of the same chemicals and with longer intervals of exposure.

To minimize the number of tubes necessary for testing each sub-strain, immediate transplants from the 0.3 per cent sodium bicarbonate suspensions were used as controls. When a poor growth was obtained in the control test, the results were discarded.

Experiments were made to determine the time concentrations of sodium bicarbonate and hexylresorcinol necessary to reduce the number of viable organisms in the majority of cultures. Effective reduction was accomplished by exposing the strains to 0.4 per cent sodium bicarbonate and to 1:150,000 hexylresorcinol for 3 hours. However, a shorter interval seemed desirable and this was achieved by exposing the cultures to 0.5 per cent sodium bicarbonate and 1:125,000 hexylresorcinol for 2 hours.

Tests made according to previously published directions (Chapman and Rawls, 1936) were used in conjunction with these tests, the combined tests being conducted as follows:

To the first row of tubes was added 0.5 cc. of 0.3 per cent sodium bicarbonate

To the second row of tubes was added 0.5 cc. of 0.5 per cent sodium bicarbonate

To the third row of tubes was added 0.5 cc. of 1:200,000 hexylresorcinol

To the fourth row of tubes was added 0.5 cc. of 1:125,000 hexylresorcinol.

Not more than 10 strains were tested at one time. A loopful of each culture to be tested was added to a tube from each of the four rows. The racks were thoroughly shaken and a loopful from each tube in the first row was streaked for about 2 cm. on blood agar to serve as a control. The racks were placed in the incubator. At the end of 1 hour, the first and third rows were removed and a loopful from each tube was streaked on blood agar alongside the control streak. At the end of the second hour, the second and fourth rows were removed from the incubator and a loopful from each tube was also streaked on blood agar. After overnight incubation of the blood agar plates, the four streaks from each culture were compared separately with the correspond-

TABLE 1

Comparison of resistance of streptococci to two different chemical agents

Reagents used: (a) sodium bicarbonate 0.3 per cent for 1 hour, 0.5 per cent for 2 hours; (b) hexylresorcinol 1:200,000 for 1 hour, 1:125,000 for 2 hours.

(A)	(B)	SERIES 1	SERIES 2	SERIES 3	SERIES 4
8+	8+	72	182	120	123
8	7	14	19	2	2
8	6		1		
7	8	9	13	3	1
7	7	46	63	91	72
7	6	16	13	5	
6	8	1			
6	7	20	10		2
6	6	34	41	47	56
6	5	12	12	11	8
6	4	1			
5	7	3			
5	6	11	4	2	4
5	5	32	15	18	20
5	4	5	5	2	4
4	6		1		
4	5	9	1		1
4	4	44	29	16	14
4	3	11	8	3	4
4	2	1			
3	4	15	10	1	5
3	3	33	29	13	14
3	2	5	2	1	1
2	3	11			
2	2	18	1		1
1	1	1			
0	0	108	92	67	67
Total strains.....		532	552	410	404
Difference	± ++	7	2		
	± +	138	97	38	37
	± 0	387 (73%)	453 (82%)	372 (91%)	367 (91%)

ing control and the results graded according to the amount of reduction of growth after exposure to the particular chemical solution. This gave four sets of results for each culture. When there was a marked reduction or complete elimination of growth, the reaction was recorded as ++ or negative. Slight reduction was recorded as +++. When there was no reduction, the reaction was recorded as ++++.

It was found simpler to add the result obtained with 0.3 per cent sodium bicarbonate to that obtained with 0.5 per cent sodium bicarbonate. Also the result obtained with 1:200,000 hexylresorcinol was added to that obtained with 1:125,000 hexylresorcinol. This gave two sets of results. One represented a combination of those obtained with the two solutions of sodium bicarbonate, while the other represented the combined results obtained with the hexylresorcinol solutions. Thus, the maximum result with either chemical was 8+ and the minimum was negative.

About 3,000 strains were examined but only 1898 of the more recent ones will be considered. Of these, both chemicals gave identical results in 75 per cent of the first series (532 strains), in 82 per cent of the second series (552 strains), and in 91 per cent of the third and fourth series comprising 814 strains (table 1). In fact, of the last 814 strains, those strains which were not in complete agreement gave results which differed only by +.

The improved correlation in the later series of experiments was explained on the basis of increased technical experience.

These results suggest that the resistance factor of certain strains of streptococci may be measured quantitatively by the methods just described.

SUMMARY AND CONCLUSIONS

A series of 1868 strains was tested by exposing each strain to 0.3 per cent sodium bicarbonate for 1 hour and 0.5 per cent sodium bicarbonate for 2 hours and testing for viability, both immediately after mixing and again after exposure. With each strain the results with the two different concentrations were added together, giving combined results ranging from 0 to 8+.

Each strain was also tested by exposing it to 1:200,000 hexylresorcinol for one hour and 1:125,000 hexylresorcinol for 2 hours and testing for viability both immediately after mixing and again after exposure. The results with the two concentrations were likewise added together.

When the combined results obtained with sodium bicarbonate were compared with those obtained with hexylresorcinol, no strain gave results differing more than ++. In fact, in 83.2 per cent of the 1868 strains, the results were exactly the same with both chemicals.

These results confirm the previous conclusion of the non-specific nature of that factor of certain smooth strains of streptococci which determines their resistance to the toxic agents used in these experiments and suggest that this non-specific resistance factor may be measured quantitatively by the method described.

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III. PRELIMINARY ATTEMPTS TO CORRELATE RESISTANCE TO CHEMICALS, ETC., WITH PATHOGENIC EFFECTS[1]

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In previous papers of this series (Chapman and Rawls, 1936, and Chapman and Curcio, 1936) it was shown that there was a relationship between the resistance of certain strains of streptococci to critical time dilutions of a number of injurious agents. In particular, it was shown that the resistance of strains to sodium bicarbonate was quantitatively parallel to their resistance to hexylresorcinol. Attempts to correlate this resistance factor with certain pathogenic factors will be described in the present paper. The strains used were similar in origin and type to those used in previous experiments.

A parallel between resistance of a streptococcus to the "bactericidal" action of diluted, fresh, defibrinated guinea pig blood and its ability to produce arthritis in rabbits had been demonstrated by Rawls and Chapman (1935). This relationship suggested the possibility that the "bactericidin" reaction, and by analogy the chemical reactions described in the two previous papers, might be correlated with other pathogenic factors.

Preliminary studies, of which the following example may be cited, suggested such a relationship. A series of 3 rabbits was inoculated intravenously with a streptococcus that reacted negatively to the "bactericidin," hexylresorcinol and bicarbonate tests. Another series of 4 rabbits was inoculated intravenously with a

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streptococcus that reacted positively to these three tests. In both series of experiments the initial dose was from 0.5 to 0.75 cc. of culture in brain heart infusion. This was increased, as tolerated, every 3 to 5 days. Rabbits inoculated with the first strain showed no apparent effects until a dose of at least 15 cc. of culture had been given. On the other hand, those rabbits inoculated with the second strain, died when the dose reached 3.5 to 6.0 cc. (table 1).

TABLE 1

Constancy of effect on rabbits of intravenously injected live cultures of streptococci

STRAIN NUMBER	RESULTS OF "BACTERICIDIN", HEXYLRESORCINOL AND BICARBONATE TESTS	RABBIT NUMBER	INITIAL DOSE	MAXIMUM DOSE	TOTAL AMOUNT INJECTED	PERIOD OF OBSERVATION	EFFECT ON RABBIT
			cc.	*cc.*	*cc.*	*days*	
8012	Negative	6	0.5	20.0	150.5	83	Survived
		9	0.75	15.0	65.75	59	Died
		12	0.75	15.0	81.25	85	Died
			Mean	16.7	99.8	76	
7842	Positive	5	0.5	3.5	6.5	24	Died (very sick in 19 days)
		8	0.5	4.5	12.0	39	Died (very sick in 32 days)
		11	0.75	6.0	19.75	32	Died
		25	0.5	4.5	18.9	70	Died
			Mean	4.6	14.3	41	

These experiments indicated that the strain that reacted positively to the *in vitro* tests was able to kill rabbits in much smaller doses than the strain that reacted negatively to these tests.

To facilitate the examination of a larger series of strains, the technic was modified by using only one injection of 5.0 cc. of brain heart infusion culture. Rabbits were injected intravenously with 20 purified sub-strains which differed in their reaction to the "bactericidin" test. The results are tabulated in table 2. Of 16 rabbits injected with sub-strains that gave ++++ "bactericidin" reactions, 14 died (87.5 per cent), while all the 4 rabbits injected with strains that were negative or only weakly positive

survived. The difficulty of finding negatively reacting strains which suspended uniformly in brain heart infusion prevented the use of a larger series of "negative" strains.

The possibility presented itself that the lethal effect of these intravenously-injected live cultures might be due, not alone to the ability of the organisms to propagate within the experimental

TABLE 2

Intravenous injection of 5.0 cc. of live brain heart infusion cultures of streptococci into rabbits (1500 ± 100 grams)

RABBIT NUMBER	ORGANISM	"BACTERICIDIN" REACTION	EFFECT OF RABBITS
47	Streptococcus, alpha	++++	Died, 10 days
48	Streptococcus, alpha	++++	Died, 10 days
49	Streptococcus, alpha	++++	Died, 2 days
50	Streptococcus, alpha	++++	Died, 10 days
52	Streptococcus, alpha	++++	Died, overnight
53	Streptococcus, alpha	++++	Died, 10 days
55	Streptococcus, alpha	++++	Died, 9 days
22	Streptococcus, alpha	++++	Died, 5 days
28	Streptococcus, alpha	++++	Died, 10 days
30	Streptococcus, alpha	++++	Died, 10 days
29	Streptococcus, alpha	++++	Died, 7 days
46	Streptococcus, alpha	++++	Survived*
54	Streptococcus, alpha	++++	Survived
26	Streptococcus, beta	++++	Died, 6 days
23	Streptococcus, beta	++++	Died, 10 days
51	Streptococcus, beta	++++	Died, 4 days
21	Streptococcus, alpha	0	Survived
27	Streptococcus, alpha	0	Survived
24	Streptococcus, gamma	++	Survived
25	Streptococcus, gamma	++	Survived

* Survived 10 days.

animal, but also to the presence of toxic factors. For this reason, it was felt that a lethal effect due to toxins alone, if such could be demonstrated, might have a greater significance in relation to streptococci isolated from persons with chronic diseases, where the symptoms often suggest toxic effects. Further experiments were undertaken with this purpose in view.

Because this type of strain only infrequently produces a soluble

toxin, any toxicity of the cultures should be connected with the bacterial cells. Consequently, washed suspensions were used in further experiments. To eliminate invasiveness killed suspensions were employed. The cells were killed with "Merthiolate" on the assumption that it produced less drastic changes than either heat or phenol. The technic adopted was as follows: A liter of brain heart infusion culture of each purified sub-strain was centrifuged and carefully decanted and drained. The sediment was suspended in 5.0 cc. of 1:1000 "Merthiolate." When sterile, 0.5 cc. was injected intraperitoneally into each of several mice.

Irregular results were obtained and very few of the suspensions produced consistent lethal effects. The experiments were repeated on rabbits with the exception that, to minimize the danger of emboli, the suspensions were filtered through cotton. However, as with mice, very few consistent lethal effects were obtained. Undoubtedly, filtering the suspensions had removed much of the bacterial mass and, possibly, the dose was too small.

Larger doses of unfiltered suspensions were prepared as follows: Pyrex bottles containing 4 liters of brain heart infusion were seeded with live cultures of streptococci and incubated overnight. The resulting growths were tested for purity, centrifuged, and the supernatants carefully siphoned off. The sediments were collected and suspended in a few cubic centimeters of 1:1000 "Merthiolate." This amount of "Merthiolate" was not lethal when injected into rabbits. Because the suspensions were extremely dense and had a strong tendency to autoagglutinate, plate counts and chamber counts were not satisfactory and nitrogen determinations were used as a basis for calculating their density. It is not intended to imply that nitrogen determinations were accepted as entirely suitable as measures of the density of the bacterial population, but duplicate determinations gave closer agreement than was obtained by counting methods.

Amounts of suspension containing 10 mgm. nitrogen were injected intravenously into rabbits weighing 1500 $\pm$ 100 grams. If a rabbit died, a smaller amount was injected into other rabbits. On the other hand, if the rabbit survived, larger amounts were

injected into other rabbits. In a few instances, the quantity of suspension available was insufficient for further tests.

Twelve rabbits died within a few minutes after inoculation, 2 died within 1 hour and 1 died within 6 hours. Because the

TABLE 3

Intravenous injection of unfiltered, killed ("Merthiolate") suspensions of streptococci into rabbits (1500 ± 100 grams)

STRAIN NUMBER	ORGANISM	1 HOUR'S EXPOSURE TO		EFFECT ON RABBITS				
		0.3 per cent bicarbonate	1-200,000 hexylresorcinol	Milligrams nitrogen injected				
				1	5	10	15	20
2710	Streptococcus, alpha	++++	++++	–	Do	Do		
2712	Streptococcus, beta	++++	++++		–	D 3		
2759	Streptococcus, alpha	++++	++++	–	–	Do		
2752	Streptococcus, alpha	++++	++++	–	D 1	Do		
2814	Streptococcus, alpha	++++	++++		Do	Do		
2618	Streptococcus, alpha	++++	++++		–	Do		
4187	Streptococcus, alpha	++++	++++		–	Do		
4120	Streptococcus, alpha	++++	++++		Do	D 2		
4378	Streptococcus, alpha	++++	++++		–	–	Do	
4444	Streptococcus, alpha	++++	++++			D ½	D ½	
4083	Streptococcus, alpha	++++	++++		–	Do –	Do	
4468	Streptococcus, alpha	++++	++++		–	Do Do		
4756	Streptococcus, alpha	++++	++++			D 3 –		
4754	Streptococcus, alpha	++++	++++		D 1 –	D ½		
4712	Streptococcus, alpha	0	0		–	Do –	–	
4472	Streptococcus, alpha	+++	++			– –	–	
4375	Streptococcus, gamma	++	++			–	–	–
2713	Streptococcus, alpha	0	0			–		

– = survived 6 days; Do = died, overnight; D = died, followed by number of days after inoculation.

suspensions were extremely dense and the tendency to autoagglutination was marked, it was thought that these lethal effects might be due to emboli or shock and should be disregarded.

Qualitative reactions with sodium bicarbonate and hexylresorcinol (Chapman and Rawls, 1936) were used as *in vitro* tests. The results of these comparisons are recorded in table 3.

It will be seen that, when 14 strains of streptococci that reacted positively (++++ or +++) to the bicarbonate and hexylresorcinol tests were injected into 17 rabbits in quantities containing 10 mgm. of nitrogen, 82.4 per cent of the animals died within 3 days. When 13 rabbits were injected with amounts of these cultures containing only 5 mgm. of nitrogen, 38.5 per cent of

TABLE 4

Comparison of in vivo and in vitro tests of smooth sub-strains of streptococci

REACTION	RESULT	MATERIAL USED FOR INJECTION	NUMBER OF STRAINS USED	NUMBER OF RABBITS INJECTED	AMOUNT INJECTED	PER CENT DIED
"Bactericidin"	++++	Live brain heart infusion cultures	16	16	5.0 cc.	87.5
	++ or 0	Live brain heart infusion cultures	4	4	5.0 cc.	0.0
Bicarbonate and hexylresorcinol	++++	Killed ("Merthiolate")	12	13	5 mgm. N	38.5
			14	17	10 mgm. N	82.4
			3	3	15 mgm. N	100.0
	++ or 0	Killed ("Merthiolate")	4	6	10 mgm. N	0.0*

* One rabbit died, but this was attributed to other factors (see text).

them died. On the other hand, when 4 strains that gave a weakly positive (++) or negative reaction to the bicarbonate and hexylresorcinol tests were injected into 6 rabbits in amounts containing 10 mgm. of nitrogen, only one died. However, it is not likely that this single lethal effect was of any significance because other rabbits injected with a similar and even larger amounts of the same suspension survived.

The agreement between these *in vivo* and *in vitro* tests is in harmony with the results previously obtained by comparison of the "bactericidin" reaction of streptococci with the effects of

injection of their live cultures into rabbits. The two sets of comparisons are summarized in table 4.

These results suggest a similarity between the properties of streptococci responsible for: (1) resistance to the "bactericidal" action of diluted, fresh, defibrinated guinea pig blood; (2) resistance to various agents, such as sodium bicarbonate and hexylresorcinol; (3) the lethal effects of live cultures; and (4) the lethal effects of killed ("Merthiolate") cultures of the strains.

The number of strains used in these animal inoculation experiments was small for two reasons. First, considerable difficulty was encountered in preparing large quantities of suspension. Second, most of the strains that reacted negatively or only weakly positive to the *in vitro* tests were of the SR or R types, leaving very few cultures available for animal experiments.

An extension of these studies is in progress using for comparison the quantitative *in vitro* tests described in the preceding paper.

SUMMARY

The intravenous injection of 5.0 cc. of live cultures of smooth streptococci that gave positive "bactericidin" reactions produced lethal effects in 14 out of 16 rabbits (87.5 per cent). Four rabbits injected with a similar amount of culture from negatively reacting smooth strains survived.

Killed ("Merthiolate") suspensions of smooth streptococci that were resistant when exposed for 1 hour to 0.3 per cent sodium bicarbonate and 1:200,000 hexylresorcinol were injected intravenously into 17 rabbits in amounts containing 10 mgm. of nitrogen. Lethal effects in 3 days were produced in 82.4 per cent of the rabbits. On the contrary, with one exception that was probably due to other factors, lethal effects were not produced in rabbits by the injection of a similar amount of suspension prepared from strains that were not resistant to bicarbonate and hexylresorcinol.

CONCLUSIONS

The results obtained with this small series of cultures suggest the advisability of further studies of the possible relationship

between: (1) the resistance to the "bactericidal" action of diluted, fresh, defibrinated guinea pig blood; (2) resistance to sodium bicarbonate, hexylresorcinol, etc.; (3) the lethal effect of live cultures; and (4) the lethal effect of killed cultures of smooth streptococci associated with suspected foci of infection of patients with chronic diseases.

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THE PURIFICATION AND CONCENTRATION OF DIPHTHERIA TOXIN

I. EVALUATION OF PREVIOUS METHODS; DESCRIPTION OF A NEW PROCEDURE

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An investigation of the chemical nature of bacterial toxins must await the isolation of these substances in a state of relative purity. It is unnecessary to emphasize the importance of such an investigation for the purpose of elucidating the nature of toxins as antigens, as substances having marked actions on living cells and tissues, and as poisons for which it may eventually be possible to find chemical antidotes. With these possibilities in mind we have undertaken to isolate, in a pure state, diphtheria toxin which is, up to the present time, the best known and most thoroughly studied of all the bacterial toxins.

Precipitation by acid. Of the various methods of purifying diphtheria toxin so far investigated, precipitation by acid seems, on the whole, to have yielded the purest products. Watson and Wallace (1924), Sedallian and Gaumont (1927) and Locke and Maine (1928, 1930) have precipitated the toxin with glacial acetic or hydrochloric acids, removing 98 to 99 per cent of the nitrogenous impurities. Koulikoff and Smirnoff (1927) obtained by the precipitation of toxin with hydrochloric acid at pH 4.7 a product reported to have a "lethal dose" of 0.0006 to 0.00028 mgm. dry weight. Koulikoff and Smirnoff (1927) and Locke and Maine (1928, 1930) have shown that the iso-electric point of the toxin is between pH 4.7 and 5.2, whereas the point of maximum precipitation of inert protein material in the toxic filtrates was between pH 3.8 and 4.6. The work of Schmidt and his col-

laborators (1930, 1931) Kjaer (1932) Bunney, Cianciarulo and Kiamil (1931) and Leonard and Holm (1933) has shown that acid precipitation causes a loss of toxicity, antigenicity and flocculating ability.

Adsorption methods. The method of purification consisting in adsorption on colloidal aluminum hydroxide and elution with sodium phosphate or other alkaline salt solutions, first used by Willstaeter for the purification of enzymes, has been applied, with modifications, to the purification of diphtheria toxin and anatoxin by Lindstrøm-Lang and Schmidt (1930), Schmidt and his collaborators (1930, 1931), Maschmann, Kuster and Fischer (1931) and Tasman and van Waasbergen (1932). Toxins and anatoxins purified by the aluminum hydroxide method show much less loss of flocculating, antigenic and toxic properties, but contain about five to ten times as much nitrogen per Lf unit as toxins purified by acid precipitation.

Besides aluminum hydroxide, other adsorbents have been used for the purification of diphtheria toxin. Abt (1928) adsorbed the toxin on a precipitate of calcium phosphate produced in the crude toxic filtrate, dissolved the precipitate in sodium citrate solution and precipitated the calcium with sodium oxalate, thus obtaining about a fifty-fold purification. Gross (1929) and Ecker and Weed (1932) have described a method of adsorption on colloidal magnesium hydroxide and elution with acid ammonium phosphate solution.

Diphtheria toxin cannot be appreciably concentrated by adsorption and elution methods because the toxin is completely eluted from the precipitates only by relatively large volumes of solution.

Precipitation by alcohol and acetone. The purification and concentration of toxin and anatoxin by precipitation with acetone, methyl alcohol, or ethyl alcohol, have been studied by Schmidt and his collaborators (1930, 1931), by Wadsworth, Quigley and Sickles (1932, 1933), by Leonard and Holm (1933), as well as by several earlier investigators. In general these methods have been found to yield impure and considerably damaged toxins.

Precipitation by metallic salts. The precipitation of diphtheria

toxin by metallic salts was first studied by Brieger and others soon after the discovery of the toxin. More recently, Smith (1932) after studying the precipitation of anatoxin by a number of metallic salts concluded that alum gave the best products as regards immunizing properties and purity. Wallace (1927), Tasman and van Waasbergen (1932), Leonard and Holm (1933) and others have reported good results from the purification of toxin and anatoxin by the use of various aluminum salts. The toxin may be dissolved from the alum precipitates by the use of sodium citrate or sodium phosphate solution. Since most of the recent studies on precipitation by metallic salts have been made on anatoxin, the data on the toxicity of toxins purified by this method are inadequate.

Purification by ultra filtration. The concentration and purification of diphtheria toxin by ultra filtration have been studied by Glenny and Walpole (1915) and more recently by Wadsworth and Quigley (1934). By this method the toxin is recovered in an unchanged state, but the degree of purification is less than that obtained by adsorption or precipitation procedures. Undoubtedly dialysis removes considerable amounts of the peptone impurities from crude toxin, but the proteoses and proteins also present, probably being of a molecular size close to that of the toxin itself, cannot be separated by dialysis or ultra filtration.

Most of the processes for purifying diphtheria toxin so far described in the literature have involved only one or two chemical manipulations. Attempts at further purification by repeated precipitations or adsorptions have generally resulted in partial or complete destruction of the toxicity and flocculating ability of the toxin. The results of the purification procedures to be described in this paper will demonstrate that diphtheria toxin may be put through a number of steps of purification without much damage if the pH of the toxin solutions is kept between 6.0 and 9.0 wherever possible and the use of organic fluids such as alcohol and acetone and of chemicals with strong oxidizing or reducing properties is avoided.

During the course of this work the Ramon flocculation test has been used as the most convenient method of determining the

amount of toxin plus toxoid in the solutions studied. The Lf unit is a rough measure of the Lo dose. Therefore the determinations based on this unit and referred to as "toxin" in this paper actually represent toxin, toxoid, and possibly other modifications. Actual measurements of toxicity must, of course, be done by measuring the M.L.D.; but comparisons of Lf units and M.L.D. in a number of purified preparations have shown that the Lf unit is a fairly reliable index of the toxicity provided no considerable proportion of toxin is changed to toxoid or other non-toxic modification during the process of purification. A change of toxin to toxoid is indicated by a falling off in the M.L.D./Lf ratio. An alteration in the serologic reactivity or "avidity" of the toxin is indicated by an increase in the Kf.

Low yields of purified toxin indicate either that the process used is inefficient, or that large amounts of toxin have been destroyed. We have considered significant only results in which 40 per cent or more of the Lf units are recovered after any one chemical manipulation used in the process of purification. When the purification procedure involves many steps low yields may be expected as the result of the accumulation of inevitable small losses at each step.

PREPARATION OF CRUDE DIPHTHERIA TOXIN

The medium used for the production of diphtheria toxin was the infusion-free peptone medium described by Wadsworth and Wheeler (1934). The composition of this medium is as follows:

	per cent
Sodium chloride	0.5
Dipotassium phosphate	0.1
Monopotassium phosphate	0.1
Magnesium sulphate	0.02
Calcium chloride	0.01
Peptone (Difco Proteose)	2.0
Sodium lactate	0.5
Glucose	0.2
Water	to 100

The directions for preparation of this medium are the same as those given by Wadsworth and Wheeler.

After about twenty lots of toxin had been prepared on this medium, it was found that the addition of 0.2 per cent of maltose somewhat increased the average strength of the toxins produced; so maltose has been added to all subsequent lots of medium. (See: Hazen and Heller, 1931, 1932; and Pope and Healey, 1933.)

The Park 8 strain was used for the production of all of the toxin. Cultures were grown on 600 cc. lots of medium dispensed in wide-mouth three-liter Erlenmeyer flasks so that the surface-area-volume ratio was 0.58 as recommended by Wadsworth and Wheeler. Cultures were incubated eight days at 34°C.

The results of toxin production on this medium have been uniformly good. In no case has a lot of toxin with an Lf value lower than 9 been obtained; the Lf value of some lots has been as high as 25 units per cubic centimeter. Generally toxins with an Lf value of 16 to 20 and an M.L.D. of 600 to 1000 when fresh and 300 to 400 after ripening were obtained.

For studies on the purification of diphtheria toxin the Wadsworth-Wheeler medium has advantages in its relatively low content or organic nitrogen (about one-half to one-third that of infusion or tryptic digest media), and in the fact that it contains no protein to complicate the purification procedures. The sterile medium gives no precipitate with acid at any pH, but after the growth of the diphtheria cultures the toxic filtrates contain proteins precipitable at pH 3.6 to 4.6.

METHODS OF PURIFICATION OF DIPHTHERIA TOXIN

Two methods used for the purification of diphtheria toxin in this laboratory have been briefly outlined in a previous paper by Eaton and Bayne-Jones (1935). Although these have now been replaced by a newer improved method, they are described in more detail here, principally because the chemical processes used in the purification give some clue as to the nature of the toxin.

Method I. Acid precipitation and ammonium sulphate fractionation

The toxic fraction was precipitated by adding 2 to 3 per cent of glacial acetic acid to the cooled crude toxin until the pH

reached 3.8, and dissolved in dilute sodium hydroxide or phosphate buffer at a pH near 8.0 to give a brown solution. Attempts at refractionation of the precipitate with acid as described by Locke and Maine (1928, 1930) yielded unsatisfactory results.

Separation of bacterial protein from toxin by means of ammonium sulphate was attempted. At one-third saturation with ammonium sulphate considerable amounts of bacterial protein were precipitated along with some toxin. The resulting precipitate dissolved in water to an opalescent greenish-brown solution from which a considerable amount of precipitate separated on standing in the ice box. This green-colored fraction flocculates poorly with antitoxin and without a definite optimum point.

Between one-third and two-thirds saturation with ammonium sulphate most of the toxin is precipitated along with small amounts of bacterial protein and proteoses. This precipitate dissolves easily in water to a clear red solution. The resulting purified toxin solution partially freed of bacterial protein remains clear indefinitely, flocculates well with antitoxin and retains its flocculating value unchanged when kept in the ice box.

The second or red fraction, containing the purified toxin, was dialyzed against running water for one or two days to remove ammonium sulphate and determinations of total nitrogen, Lf units and M.L.D. were made. For results see table 1.

One experiment using this method for the purification of anatoxin gave results similar to those obtained with toxin.

Method II. Fractionation with ammonium sulphate, adsorption on aluminum hydroxide, elution with sodium phosphate solution, refractionation and concentration of the eluted toxin with ammonium sulphate

It was considered desirable to remove from the crude toxin as much of the bacterial protein as possible before adsorption on aluminum hydroxide. This was accomplished by adding 233 grams of ammonium sulphate (one-third saturation) to each liter of crude toxin, keeping the pH near 7.0 by the addition of a few cubic centimeters of N/1 sodium hydroxide. The resulting precipitate was filtered off on paper and dissolved in water to a

greenish-yellow solution. This fraction contains 10 to 20 per cent of the total Lf units in the original toxin, some proteose and considerable protein. It flocculates slowly with antitoxin without a sharp optimum point.

TABLE 1

Summary of toxin preparations purified by various methods

METHOD OF PURIFICATION	Lf UNITS PER CC.	M.L.D. PER CC.	NITROGEN PER CC.	NITROGEN PER Lf UNIT	NITROGEN PER M.L.D.	YIELD OF Lf UNITS	PURIFICATION FACTOR
			mgm.	*mgm.*	*mgm.*	*per cent*	
Method I	70	400*	0.035	0.0005	0.00008	20	260
Method I	145	3,000	0.185	0.0013	0.00006	14	100
Method I (anatoxin)	165		0.160	0.0010		15	130
I and lead acetate precipitation	120		0.110	0.0009		11	144
I and II	38		0.044	0.0012		10	108
I and II	140	3,000	0.100	0.0007	0.000033	9	186
I and II, Pb Ac supernatant	70		0.069	0.0010		5.4†	130
I and II, Pb Ac precipitate	70		0.050	0.0007		3.6†	186
Method II	90	2,000	0.62	0.0067	0.00033	15	19
Method II	128	7,500	0.64	0.0050	0.00008	30	26
Method II	140	4,000	0.65	0.0047	0.00015	35	28
II and phosphotungstic acid	120	2,000	0.100	0.00083	0.00005	15	158
II and phosphomolybdic acid	120	2,500	0.165	0.00130	0.00006	12	100
II and cadmium chloride	140	3,000	0.110	0.00080	0.00004	15	162
Crude toxin (average)	15	(300–800)	2.40	0.130		(100)	0

* M.L.D. this preparation estimated by intradermal test; all others directly by subcutaneous injection.

† Yields calculated on basis of 60 per cent of Lf units in supernatant and 40 per cent of Lf units in precipitate, see text.

To the filtrate from the first fraction 175 grams of ammonium sulphate per liter were added, bringing the concentration to seven-twelfths of saturation. The pH was readjusted to 7.0 with sodium hydroxide. The heavy, sticky precipitate of proteoses and toxin was centrifuged down and dissolved in an amount of water representing about one-twentieth the volume of the original

toxin. The resulting dark reddish brown solution contains 70 to 90 per cent of the original total Lf units of toxin in an unaltered form which flocculates quickly and sharply with antitoxin.

The adsorption on aluminum hydroxide and elution with 2.4 per cent disodium phosphate was carried out by the method described by Tasmann and van Waasbergen (1932), except that it was done on the second ammonium sulphate fraction diluted so as to contain about 30 Lf units per cubic centimeter instead of on the crude toxin.

The solution of toxin eluted from the aluminum hydroxide generally contains about 30 Lf units per cubic centimeter, a solution too dilute for further study of the toxin. Since considerable amounts of inert protein and proteose as well as toxin are adsorbed by the aluminum hydroxide and eluted again it was considered advisable to attempt the separation of these fractions by a second ammonium sulphate fractionation by which at the same time a concentration of the toxin could be accomplished.

The phosphate solution of eluted toxin was therefore fractionated with ammonium sulphate at one-third and two-thirds saturation as described above.

Most of the toxin was recovered in the second ammonium sulphate precipitate. The solution of this precipitate was dialyzed against running water to remove the ammonium sulphate and on the resulting light-yellow solution of purified toxin determinations of Lf, M.L.D. and total nitrogen were done. The results are summarized in table 1.

Further purification of toxins prepared by methods I and II

As indicated in the description of method I, the purified toxin prepared by acid precipitation and ammonium sulphate fractionation appears to consist almost entirely of toxin and bacterial protein. Separation of the toxin from the protein was therefore attempted by means of adsorption on aluminum hydroxide, further fractionation with ammonium sulphate, and precipitation with lead acetate.

Two preparations of toxin purified by method I were diluted with water, adsorbed on aluminum hydroxide, eluted with phos-

phate and fractionated with ammonium sulphate in the manner described under method II. The second ammonium sulphate fraction containing the toxin was dialyzed. The resulting colorless solutions of highly purified toxin flocculated well with antitoxin and were very toxic for guinea pigs. These preparations were practically free of proteose but still contained protein.

Experiments using neutral lead acetate as a protein precipitant were carried out on a preparation of toxin obtained by method I and also on the preparation described in the previous paragraph. In no case was any appreciable amount of purification obtained by the lead acetate treatment and the toxin appeared to be unchanged by fractional precipitation with this reagent. The results of these experiments are summarized in table 1.

The preparations of toxin obtained by method II, although apparently relatively free of bacterial protein, were found to contain considerable amounts of a substance resembling proteose in its properties, and these preparations were more impure than those obtained by acid precipitation. Simple acid precipitation of the toxin from these preparations gave unsatisfactory results. However, it was found that, by precipitation with phosphotungstic acid with careful adjustment to pH 4.8 to 5.0 the toxin could be separated from most of the proteose impurities, thus producing a considerable additional purification.

The phosphotungstic acid toxin precipitate was suspended in water and decomposed by adding 5 per cent barium hydroxide solution until the pH reached 9.4, centrifuging and removing the excess of barium from the supernatant as insoluble $BaSO_4$. The resulting clear colorless solution of toxin showed a slightly increased flocculation time, probably due to the action of the acid, but was quite toxic for guinea pigs. Similar results were obtained using phosphomolybdic, tungstic or molybdic acid in place of phosphotungstic acid.

In order to obtain a purified toxin unchanged by the action of acid, an attempt was made to separate the proteins and toxin from the proteoses in the preparations of method II by precipitation with metallic salts in neutral or alkaline solution. Of the various metal salts investigated, cadmium chloride appeared to

be the most satisfactory for this purpose. Details of this method are omitted here because a more successful modification was later worked out. The toxin obtained by this method is of about the same degree of purity as that obtained with phosphotungstic acid, but the cadmium chloride preparation flocculates more readily and is somewhat more toxic.

Summary of results of the various methods of purification

The data on a number of toxin preparations purified by the methods just described are presented in table 1. The figures for crude toxin included in the table represent the average of a number of samples. Because of the uniformity in Lf units and nitrogen content of the lots of crude toxin prepared during the course of this work, the calculations of the purification factors given in the last column of table 1 were based on an average figure for crude toxin. These factors represent the ratio:

$$\frac{\text{Mgm.N per cc./Lf units per cc. (crude toxin)}}{\text{Mgm.N per cc./Lf units per cc. (purified toxin)}}$$

It may be seen from the figures in the table that the preparations obtained by method I are about five times as pure as those obtained by method II. Adsorption on aluminum hydroxide, fractionation with ammonium sulphate, and precipitation with lead acetate do not materially increase the purity of toxins prepared by method I. On the other hand, precipitation of the toxic fraction by phosphotungstic acid, phosphomolybdic acid or cadmium chloride from the preparations obtained by method II increases the purity of these toxins to a degree comparable with the preparations obtained by method I.

In general, the yields of Lf units obtained in method II alone or in combination with the other chemical manipulations described, are greater than those obtained by the various modifications of method I. The toxicity of the acid-purified preparations was not studied in great detail because it was evident from this work and the work of others that the toxicity is often partially destroyed by the action of acid.

Highly toxic products were consistently obtained by the use

of method II, and the toxicity per milligram of nitrogen in these preparations could be somewhat increased by using the protein precipitants described to separate out the toxic fraction.

The purified toxins obtained by methods I or II alone are generally colored red, pink or brown. Further purification removes these colored impurities so that the most highly purified toxins are generally colorless or very light yellow.

None of these purification procedures is entirely satisfactory. Acid precipitation followed by ammonium sulphate fractionation gives highly purified but chemically altered products. Adsorption on aluminum hydroxide does not, by itself, produce enough purification; and the difficulty of obtaining large quantities of suitable aluminum hydroxide cream limits the amount of toxin that can be worked up by this method. The use of phosphotungstic acid to purify the aluminum hydroxide preparations also damages the toxin.

Method III. New procedure—precipitation with alum and cadmium chloride

With the object of devising a simpler, more efficient method of purification applicable to large scale operations, experiments with metal salt precipitation were carried out. From the previous results it was apparent that the use of acid should be omitted in order to avoid damage to the toxin. It was found that the toxic protein fraction was precipitable by metal ions in a neutral or alkaline solution under appropriate conditions, and that most of the non-protein impurities were left in solution. The new purification procedure described below was then worked out.

Preliminary experiments. After a number of experiments on the precipitation of diphtheria toxin from the crude filtrates by various metal salts, aluminum ammonium sulphate and cadmium chloride were selected as the two best suited to the purification of toxin with a minimum of damage. Experiments on the best conditions for the use of these reagents were then undertaken.

It was found that the phosphate present in the Wadsworth-Wheeler medium interfered with metal salt precipitation of the toxin. Under controlled conditions nearly all of the phosphate

could be precipitated as calcium phosphate without much adsorption of toxin by the precipitate. From the resulting filtrates the toxin was then easily precipitated by ammonium alum or by cadmium chloride.

The small amount of toxin (about 10 per cent) adsorbed by the calcium phosphate precipitate is about twice as pure as the crude toxin, but considerable bacterial protein is also adsorbed on this calcium phosphate precipitate. This "protein" fraction is colored green and resembles the green fraction obtained in methods I and II described in a previous section.

By precipitating the calcium phosphate filtrates with cadmium chloride and treating the precipitate with 2.4 per cent disodium phosphate, a purified toxin containing 0.011 mgm. N/Lf unit, or a fifteen-fold purification, was obtained; but the yields were only 30 or 40 per cent.

Somewhat better yields were obtained by alum precipitation but the toxin could be completely precipitated only at a pH near 5.0, thus exposing it to acid destruction. It was then found that by carrying out the alum precipitation in the presence of a concentration of ammonium sulphate equivalent to one-third saturation, the toxin was precipitated near pH 6.0 in a yield of 70 to 80 per cent. In this procedure the bacterial protein salted out by the ammonium sulphate was separated by filtration before the alum was added.

Washing of the alum precipitates with water was found to be useful in removing about 30 per cent of the nitrogenous impurities without loss of toxin. The alum precipitates were then dissolved in sodium citrate solution as described by Smith (1932). The degree of purification obtained after the alum precipitation was ten- to fifteen-fold.

Further purification of the citrate solutions of the alum precipitates could be accomplished by salting out with ammonium sulphate, by precipitation with acid, or by precipitation with cadmium chloride. The cadmium chloride precipitation was found preferable because it gave the best purification with good yields and little damage to the toxin.

An experiment with stabilized old crude toxin showed that very

little change of toxin to toxoid, as evidenced by the M.L.D./Lf ratio, occurred in the alum and cadmium chloride method of purification. This is demonstrated in the following summary:

Crude toxin—15 Lf units per cubic centimeter; 300 M.L.D. per cubic centimeter; ratio M.L.D./Lf = 20

Same toxin after alum and cadmium chloride purification—200 Lf units per cubic centimeter; 4000 M.L.D. per cubic centimeter; ratio M.L.D./Lf = 20

General procedure. The purification procedure finally worked out is summarized below:

1. To the crude toxic filtrate add 18 cc. of 20 per cent calcium chloride solution per liter of toxin; heavy precipitate of calcium phosphate. Let stand 15 minutes and filter through folded paper.

2. To the filtrate add gradually 233 grams of ammonium sulphate per liter (one-third of saturation), keeping the pH near 7.0 by the addition of a few cubic centimeters of N/1 NaOH. Let stand one hour in refrigerator. Filter off precipitate on folded paper.

3. To the cooled filtrate in a large bottle add 70 cc. of 10 per cent aluminum ammonium sulphate per liter with good shaking. The pH should be about 6.0. Stopper and let stand overnight in the refrigerator. Filter off the heavy precipitate in a Buchner funnel.

4. Grind precipitate with water in a mortar and suspend in a volume of water one-third the volume of the original crude toxin. Let stand several hours in the refrigerator. Filter on Buchner funnel.

5. Dissolve the precipitate in a 2 to 4 per cent solution of sodium citrate using one-twentieth the volume of the crude toxin. If precipitate fails to dissolve completely add a little more 20 per cent sodium citrate solution. Determine Lf-value.

6. Dilute the citrate solution of the alum-precipitated toxin so that it contains 100 to 120 Lf units per cubic centimeter. Adjust citrate concentration to optimum for cadmium precipitation (see below). Add 5 per cent $CdCl_2$ solution until a moderately heavy precipitate forms and pH is about 6.0. One-half to one volume of $CdCl_2$ is required, depending on citrate concentration (see

below). Let stand two hours in refrigerator and centrifuge down cadmium toxin precipitate.

7. Wash precipitate with a volume of water equal to toxin solution before $CdCl_2$ precipitation and filter on Buchner funnel. Treat precipitate with one-fourth to one-half volume of 2 per cent Na_2HPO_4 or 1 per cent $NaHCO_3$ and let stand for several hours or overnight in refrigerator with occasional mixing.

8. Centrifuge down at high speed the insoluble cadmium phosphate or carbonate. The red or red-brown supernatant contains the concentrated and purified toxin.

The chemical principles involved in this purification procedure are essentially the separation of small amounts of protein from large amounts of proteoses and peptones without the use of acid. Part of the inactive proteins are removed at the start by adsorption on calcium phosphate and salting out with ammonium sulphate. The remaining bacterial proteins mixed with a residuum of proteose are in the toxic protein fraction precipitated by the metal salts.

The effect of citrate concentration on the cadmium chloride precipitation

After several experiments it was found that some of the less pure cadmium precipitates dissolved completely in the sodium phosphate solution and this made separation of the cadmium from the toxin very difficult. The chemistry of this cadmium precipitation is not at present thoroughly understood. Apparently high concentrations of sodium citrate prevent the precipitation of certain alum-proteose compounds by the cadmium chloride. In the presence of lower concentrations of citrate these proteose impurities are carried down in the precipitate and form a compound with the cadmium which dissolves in sodium phosphate solution.

With any one lot of alum-precipitated toxin, the greater the citrate concentration, the larger the volume of 5 per cent cadmium chloride required to precipitate the toxin. Precipitates formed in the presence of low citrate concentrations are impure and soluble in phosphate; but the yields of Lf units are high. With

higher concentrations of sodium citrate the yields are lower but the products are purer, the cadmium compounds formed in the presence of phosphate are insoluble and the toxin goes into solution. These differences in precipitation conditions apparently have no effect on the M.L.D./Lf ratio of the products.

The optimum concentration of sodium citrate for cadmium chloride precipitation varies with different lots of alum-precipitated toxin but is generally between 2 and 4 per cent. For this reason it is advisable to work with large pooled lots of the alum precipitates and determine in advance by experiments on small samples the optimum proportions of sodium citrate and cadmium chloride.

In some cases the conditions for high yields with nearly maximum purification are such that the cadmium precipitates partly dissolve or tend to form a colloidal suspension in phosphate. It has been found better in recent experiments to use 1 to 2 per cent sodium bicarbonate to decompose the cadmium-toxin precipitates. The cadmium carbonate formed does not tend to dissolve.

Sometimes the purified toxins give a positive test for cadmium with sodium sulphide, in spite of all precautions to precipitate the metal. These preparations are freed of cadmium by treating the toxin solution with about one-fourth volume of a solution containing 1.25 per cent $Ba(OH)_2$ and 1.25 per cent $BaCl_2$. The pH is then adjusted to 9.2 with $Ba(OH)_2$ and the resulting gelatinous precipitate of barium carbonate centrifuged down and washed with a little water. The barium is then removed from the combined washings and supernatant by adjusting the pH to 7.0 with sulphuric acid. Very little toxin is lost in this process.

Results of the alum and cadmium chloride purification method

The method described in the previous section is well adapted to the purification of diphtheria toxin in large quantities. Crude toxin in lots of 8 liters or more may be worked up by the alum precipitation method, and the citrate solutions of several lots of alum precipitates then combined and precipitated with cadmium chloride. In all, over 100 liters of crude toxin have been purified by the method described, and about 6 liters of concentrated and

purified toxin solution containing 150 to 200 Lf units per cubic centimeter thus obtained.

The data on two pooled lots of purified toxin, and several smaller lots, are summarized in table 2. The yields in Lf units average somewhat over 50 per cent and the toxicity of the purified products is high as shown by the M.L.D./Lf ratio in the next to the last column. This ratio for crude toxin is generally between 20 and 40. The degree of purification is nearly as great as that obtained by the method I described in the previous sections, and greater than that obtained by aluminum hydroxide adsorption. The purification factors calculated from the milligrams of nitrogen per Lf unit in crude and purified toxins are about 65 for lot 1 and 40 for lot 2.

TABLE 2

Diphtheria toxin purified by the alum and cadmium chloride method

PREPARATION NUMBER	Lf UNITS PER CC.	NITROGEN PER Lf UNIT	M.L.D. PER CC.	NITROGEN PER M.L.D.	RATIO M.L.D. PER Lf	YIELD OF Lf UNITS
		mgm.		*mgm.*		*per cent*
21	200	0.0015	4,000	0.000085	20	59
22	150	0.0022				42
271	190	0.0019	5,000	0.000075	26	56
273	160	0.0012	4,000	0.000048	25	47
27	140	0.0017	5,000	0.000048	35	52
Lot 1	205	0.0018	8,000	0.000050	38	51
Lot 2	145	0.0028	4,500	0.000090	32	52

The flocculation time of these preparations is not appreciably increased and all give a sharp optimum point of flocculation with antitoxin.

The Lr/1000 dose of purified toxin lot 1 was determined by the usual intradermal method on guinea pigs. When mixed with 1/1000 A.T.U., 0.000007 cc. of toxin gave a minimal skin reaction. Hence this toxin contained 142,000 Lr/1000 doses per cubic centimeter or about 142 Lr doses per cubic centimeter as compared with 205 Lf units and 8000 M.L.D. per cubic centimeter, a relationship between combining power, flocculating value and toxicity about the same as that found in the general run of crude toxins.

Because the alum and cadmium chloride method makes avail-

able large amounts of relatively unchanged and considerably purified diphtheria toxin, preparations such as those described in table 2 are valuable materials for studies on the further purification of the toxin and investigations of its chemical nature.

Further purification of the alum-cadmium preparations

A chemical investigation of the purified toxin solutions described in the previous section showed that these contained, besides inorganic salts, for the most part (1) a group of coagulable substances containing nitrogen, and (2) a group of non-coagulable substances containing nitrogen. The substances in the coagulable portion apparently consist of a mixture of toxic protein and non-toxic bacterial proteins. Those in the non-coagulable part are mostly proteose impurities not completely removed by the purification process.

It was found that a repetition of the alum and cadmium chloride procedure, previously described, on the partially purified toxins produced some additional purification. The resulting products were more toxic per milligram of nitrogen than those obtained by any other previous method but the yields were low. Later it was found that loss of toxin could be avoided by omitting the second alum precipitation which was actually inessential to the purification.

An investigation of the conditions for the second cadmium chloride precipitation showed that the best results were obtained by precipitating the toxin in the presence of about 2 per cent of sodium citrate. The optimum concentration of citrate varies somewhat for different lots of partially purified toxin and should be determined first on small samples. The cadmium precipitates may be decomposed with 1 per cent solutions of either sodium phosphate or sodium carbonate. By using small volumes of these reagents relatively concentrated solutions of the purified toxin may be obtained.

The results of the purification procedures described in this section are summarized in table 3. The starting materials for some of these products were the partially purified toxins numbered lots 1 and 2 in table 2 and similarly numbered in table 3. The

preparation designated F in table 3 was made directly from a lot of fresh toxin by one alum precipitation and two precipitations with cadmium chloride.

It may be seen from the results tabulated that the method just described yields unaltered toxins of high purity. The degree of purification in both M.L.D. and Lf units per milligrams of nitrogen is over 200 fold and the yields 20 to 30 per cent.

TABLE 3

Highly purified diphtheria toxins obtained by further purification of alum-cadmium chloride preparations

LOT NUMBER	Lf UNITS PER CC.	M.L.D. PER CC.	NITROGEN PER Lf	NITROGEN PER M.L.D.	YIELD OF Lf UNITS
			mgm.	*mgm.*	*per cent*
1	160	4,000	0.00067	0.000027	11
1a	100	2,000	0.00046	0.000025	18
1b	260	6,000	0.00077	0.000033	20
1c	110		0.00070		16
2	250		0.00095		45
2a	340		0.00077		20
2b	600	14,000	0.00050	0.000021	35
2c	420	9,500	0.00049	0.000021	30
F	300	10,000	0.00055	0.000016	30
F1	420	12,500	0.00055	0.000019	20

DISCUSSION

The toxin prepared by the methods described in this paper is purer and apparently more toxic than the preparations obtained by Locke and Maine (1928, 1930) and Koulikoff and Smirnoff (1927) by acid precipitation at the iso-electric point. These authors gave very meagre data on the toxicity and flocculating properties of their highly purified toxins, and the methods described by them have subsequently been found to cause considerable damage to both toxin and anatoxin.

The difficulties of obtaining sufficient quantities of purified toxin for adequate chemical investigation have already been noted. From 100 liters of crude toxin only 1 or 2 grams of highly purified and active toxin are obtainable. However the method of purification with alum and cadmium chloride in the presence of sodium

citrate, as described in this paper, appears to be limited in its scale of operation only by the facilities for preparing crude toxin, and the size of the apparatus available for handling the toxin during the purification process.

SUMMARY

Various methods of purifying diphtheria toxin are compared and the objections to the use of acid in any purification procedure are noted.

A new method of purifying diphtheria toxin by precipitation with aluminum ammonium sulphate and cadmium chloride is described.

By this method purified toxins containing 0.0005 mgm. N per Lf unit and 0.00002 mgm. N per M.L.D. have been obtained.

Certain modifications of older methods such as acid precipitation and aluminum hydroxide adsorption gave lower yields of purified toxins containing also about 0.0005 to 0.0010 mgm. N per Lf unit, but these preparations had a lower toxicity than those obtained by the new method.

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THE PURIFICATION AND CONCENTRATION OF DIPHTHERIA TOXIN

II. OBSERVATIONS ON THE NATURE OF THE TOXIN

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In the previous paper (Eaton, 1936) a new method of purifying diphtheria toxin was described. The present paper will deal with a few additional purification experiments that have a bearing on the nature of the toxin, and with the chemical and immunological properties of the purified toxins obtained by these procedures and those previously described.

In its chemical behavior diphtheria toxin has long been regarded as a protein-like substance. The results of the present work tend to confirm this view, and also indicate that the toxic, antigenic and serologic properties of diphtheria toxic filtrates are due to a single substance which, under certain conditions, may undergo physical or chemical modification.

The purification of diphtheria toxin by metal salt precipitation (method III) has yielded products of a purity and activity high enough to make possible a direct chemical investigation of the nature of the toxic substance. From the limited amount of material so far available it has been possible to obtain definite evidence that the toxin is a protein having rather characteristic chemical properties. The results of the present work also indicate that the toxic protein is serologically and chemically distinct from the other proteins of the diphtheria bacillus.

THE NATURE OF DIPHTHERIA TOXIN AS INDICATED BY THE GENERAL PRINCIPLES AND CHEMICAL REACTIONS INVOLVED IN ITS PURIFICATION

All of the methods that can be successfully used in the purification of diphtheria toxin involve reactions more or less char-

acteristic of proteins. If diphtheria toxin were a complex carbohydrate, a lipoid, or some simpler organic substance which might under certain conditions be adsorbed by protein precipitates, several stages of purification involving different protein precipitation reactions should eventually separate the protein from the toxin. That this cannot be done is becoming more evident as work on the purification of diphtheria toxin progresses.

The purification of toxin involves the separation of the protein fraction from the chemically similar proteoses and peptones and the separation of the various protoplasmic fragments of bacteria in the protein fraction from the toxin. The methods which have been applied to these problems are summarized below.

Acid. Iso-electric point precipitation of the toxic protein fraction.

Negative ions in acid solution. Toxin is precipitable at a pH near 5.0 by various negative ions or groups such as those of phosphotungstic, phosphomolybdic, tungstic, molybdic, flavianic, picric, trichloracetic and nucleic acids, and may be recovered from some of the precipitates by appropriate treatment with alkali.

Salting out. Fractional precipitation with ammonium sulphate may be used partially to separate the toxin, precipitable at 0.4 to 0.7 saturation, from some of the proteins, proteoses and peptones.

Organic fluids. Alcohol and acetone precipitate diphtheria toxin in an altered state along with much proteose and peptone.

Metal salts. Toxin is precipitated in the pH range between 6.0 and 8.0 by metallic salts such as zinc chloride, cadmium chloride, ferric chloride, ferrous sulphate, lead acetate, silver nitrate, mercuric chloride, copper sulphate, uranyl acetate, aluminum sulphate and ammonium alum. From the precipitates produced by some of these salts the toxin is readily recovered by appropriate chemical treatment.

Adsorption. It was found that certain impurities and very little toxin were adsorbed by small quantities of adsorbing agents such as charcoal, kaolin, calcium phosphate, magnesium hydroxide and aluminum hydroxide. With larger quantities of ad-

sorbent both toxin and some impurities were adsorbed. The adsorption effects observed depended largely on the pH and the amount and character of salts in solution.

Tryptic digestion. This destroys partially purified toxin in the same way as it does crude toxin. Consequently this method cannot be utilized to remove foreign protein from the toxin.

Dialysis of toxin solutions in collodion bags has been used to remove salts and peptones from these solutions but most of the proteoses and proteins are retained inside the membrane.

PREPARATION OF HIGHLY CONCENTRATED AND PURIFIED DIPHTHERIA TOXIN

About forty liters of crude toxin were purified by the method II described in the previous paper (Eaton, 1936). This purification was done in several separate lots, and in each of these lots after adsorption on $Al(OH)_3$ the toxic fraction was precipitated, first with phosphotungstic acid and then with cadmium chloride. The final products contained 0.00053 to 0.00085 mgm. of nitrogen per Lf unit, and the purification factors were 153 to 250.

For the purpose of investigating the chemical nature of the toxin one of these preparations was evaporated *in vacuo* at a temperature of 25°C. to about one-tenth of its original volume. The resulting solution contained 1440 Lf units and 10,000 M.L.D. per cubic centimeter, flocculated in two minutes with antitoxin in diluted solutions containing 72 Lf units per cubic centimeter, and contained 0.75 mgm. of nitrogen per cubic centimeter or 0.0005 mgm. of nitrogen per Lf unit and 0.000075 mgm. of nitrogen per M.L.D. The chemical tests on this sample of toxin will be given in a later section of this paper.

STABILITY OF PURIFIED TOXIN

Three of the preparations obtained as described above were combined and stored in a dark refrigerator for eight months. At the end of this time the toxin solution showed no visible evidence of change. The determinations of toxin content and total nitrogen after the period of storage are summarized: Lf per cubic

centimeter, 180; Kf at 25°C., 5 minutes; M.L.D., 3000 per cubic centimeter; milligram N per cubic centimeter, 0.123; milligram N per Lf unit 0.00072; milligram N per M.L.D., 0.00005.

From these results it is evident that toxin in this state of purity remains unchanged and is highly toxic eight months after being purified, and over one year after the production of the crude toxin from which it was prepared. Similar results have been obtained with other samples of purified toxin after storage for six to eight months.

DIFFERENTIAL ADSORPTION EXPERIMENTS WITH PURIFIED TOXIN

In an attempt to remove the impurities from the toxin solutions obtained by method III, described in the previous paper, the effects of adding to them various amounts of adsorbents such as kaolin, charcoal, or colloidal magnesium hydroxide were studied.

1. Differential adsorption with kaolin

It was found that kaolin served as a useful adsorbent for reducing the proteose content of preparations containing considerable amounts of this substance. A protocol of an experiment on the toxin designated lot 2 in table 2 in the previous paper demonstrates this effect.

Milligrams non-coagulable N per cubic centimeter in toxin before kaolin treatment: 0.300.

a. Non-coagulable N in toxin solution after adding 10 per cent of dry kaolin and filtering: 0.140 mgm. N per cubic centimeter. Reduction of non-coagulable N: 54 per cent.

Kaolin treatment reduced Lf units from 145 per cubic centimeter to 116 per cubic centimeter, a loss of 20 per cent by adsorption of toxin to kaolin.

b. Non-coagulable nitrogen in toxin solution after adding 20 per cent of dry kaolin and filtering: 0.080 mgm. N per cubic centimeter. Reduction of non-coagulable N: 73 per cent.

Kaolin treatment reduced Lf units from 145 per cubic centimeter to 112 per cubic centimeter, a loss of 23 per cent by adsorption of toxin to kaolin.

From these results it may be seen that the proteose substances removed on the kaolin are not the toxin. Although a considerable purification is produced, complete removal of the non-coagulable nitrogen cannot be effected by this method.

2. Differential adsorption with charcoal

The adsorption effects of charcoal in the purified toxin solutions differ from those of kaolin. Weights of Darco animal charcoal equal to about 0.5 per cent of the volume of the solution decolorize the purified toxin solutions without adsorbing much toxin. Some coagulable protein is removed but no great reduction of the milligrams of nitrogen per Lf unit can be brought about in this way.

3. Differential adsorption with magnesium hydroxide

Studies on the fractional adsorption by magnesium hydroxide of the constituents of the coagulable protein portion of the purified toxin revealed that at least a partial separation of these proteins from each other is possible by this method. Further investigation will be necessary before the details of this work can be published; but, since this procedure was used as a step in obtaining some of the highly purified toxins described in this paper, a brief resumé of the results is included here.

Colloidal magnesium hydroxide added in small quantities adsorbs the red substance from the toxin solutions leaving the toxin and the colorless proteins in solution. It acts as a decolorizing agent only for purified toxin solutions.

With larger quantities of magnesium hydroxide under various conditions the purified toxin may be divided into fractions having comparable Lf values but showing differences in (1) the content of protein precipitated by acid in the presence of one per cent of sodium phosphate, (2) flocculation time and M.L.D./Lf ratio, and (3) the content of bacterial protein detected by precipitin tests. (For details of these tests see a later section of this paper.)

From the results of the magnesium hydroxide adsorption experiments it was concluded that the protein fraction containing

the toxin consisted of several proteins differing somewhat in their chemical behavior.

PURIFICATION OF TOXIN BY A MULTIPLE-PRECIPITATION PROCESS COMBINING SEVERAL DIFFERENT CHEMICAL PROCEDURES

The results described in the previous paper indicate that it is possible to obtain toxins of the highest purity by successive precipitations with alum and cadmium chloride. That the use of a greater number and variety of chemical procedures in the purification process is of no advantage may be seen from the results described below.

On about 10 liters of crude toxin the following successive chemical procedures were carried out as described in this and the previous paper.

1. Precipitated with acid. 2. Fractionated with ammonium sulphate.

3. Adsorbed on aluminum hydroxide and eluted with 2.4 per cent Na_2HPO_4.

Purity of resulting product: 0.00120 mgm. N per Lf unit.

4. Fractionated with ammonium sulphate. 5. Precipitated with alum and dissolved in sodium citrate. 6. Precipitated with $CdCl_2$.

Purity of resulting product: 0.00058 mgm. N per Lf unit.

7. Precipitated with phosphotungstic acid, dissolved in $Ba(OH)_2$.

8. Precipitated with cadmium chloride.

Purity of resulting product: 0.00053 mgm. N per Lf unit.

The final product still retained considerable toxicity having an Lf value of 125 units per cubic centimeter and an M.L.D. of 1200 per cubic centimeter; but it was not purer than the preparations already described.

The results of this experiment indicate that it is impossible to obtain diphtheria toxin containing less than about 0.00050 mgm. of nitrogen per Lf unit by any of the methods so far described, used either alone or in combinations.

THE PROTEIN CONTENT OF CRUDE AND PURIFIED TOXINS

Measurements of the nitrogen in the precipitates produced by adding 5 to 10 per cent of trichlor-acetic acid to known volumes

of toxin solution were considered to represent the protein nitrogen. The proteoses, peptones and other non-protein substances in these solutions are not precipitated by trichlor-acetic acid. Watson and Wallace (1924) and Watson and Langstaff (1926) measured the protein nitrogen precipitated by one per cent acetic acid and heat in their crude toxin preparations. This method as applied to our crude and purified toxins has yielded figures for the protein nitrogen that are obviously too low because of the incomplete precipitation of the protein. Yet the amounts of protein nitrogen determined by the trichlor-acetic acid method in our crude toxins appear to be, in general, somewhat lower than those reported by Watson and Wallace who used a different medium.

The Wadsworth-Wheeler medium when sterile, gives no measurable amount of precipitate with trichlor-acetic acid. After the growth of the diphtheria cultures and the formation of toxin, the Berkefeld filtrates contain 1 to 2 per cent of the total nitrogen as protein nitrogen. This varies considerably with different samples of toxin, and the protein content of the filtrates probably depends on the amount of autolysis during the growth of the cultures and the amount of protein hydrolysis before and after filtration. The figures given in table 1 for samples of fresh and old crude toxins represent nearly the extremes of variation among the lots of toxin prepared by us. It may be seen from the last column in the table that the amount of protein nitrogen per Lf unit is nearly three times as great in one sample of crude toxin as it is in the other.

The toxin fractions adsorbed on calcium phosphate, and precipitated by one-third saturation with ammonium sulphate contain more protein nitrogen per Lf unit than do the original crude toxins. The partially purified toxins contain one-half to one-fifth as much protein per Lf unit as the crude toxins.

With further purification of the toxin the proportion of protein to Lf units appears to approach a constant value between 0.0004 and 0.0005 mgm. of protein nitrogen per Lf unit. In the most highly purified preparations 80 to 90 per cent of the nitrogen is in the form of protein and the residue of non-protein nitrogen

appears to be mostly in a proteose precipitated by tannic acid. The total nitrogen per Lf unit in these preparations is less than 0.5 per cent of the total nitrogen per Lf unit in the crude toxin.

TABLE 1

Protein nitrogen content of diphtheria toxin at various stages of purification

LOT NUMBER	METHOD OF PURIFICATION*	Lf UNITS PER CUBIC CENTIMETER	TOTAL N	PROTEIN N	PROTEIN N	PROTEIN N PER Lf UNIT
			mgm. per cc.	*mgm. per cc.*	*per cent*	*mgm.*
	Crude toxin (old)	18	2.40	0.0237	1	0.0013
	Crude toxin (fresh)	14	2.40	0.0490	2	0.0035
	"Protein" fraction 1/3 saturated ammonium sulphate	6		0.0480	<5	0.0080
	Green fraction adsorbed to calcium phosphate	7	0.238	0.0126	5	0.0018
2	Method III	145	0.400	0.100	25	0.00070
2a	III	390	0.390	0.197	50	0.00051
2a	Same, K (10 per cent)	330		0.155		0.00046
2a	Same, K (20 per cent)	270		0.136		0.00051
2a	Same, K (10 per cent), C	340	0.249	0.155	65	0.00046
2b	III, K, Mg	115	0.106	0.0698	66	0.00061
2c	III, K, Mg	135	0.095	0.0632	67	0.00047
2d	III, K, C	600	0.294	0.252	87	0.00042
1	III, Mg	200	0.134	0.0993	74	0.00050
F	III, Mg	420	0.238	0.190	80	0.00045
17	II†	180	0.123	0.086	70	0.00050
M	‡	125	0.066	0.058	85	0.00046

* Explanation of symbols, C = charcoal adsorption, K = kaolin adsorption. Figures in parentheses after K show amount of kaolin used for adsorption of proteose impurities; note that protein N is unaffected by adsorption, Mg = adsorption on magnesium hydroxide.

† Purification by the method II described in the previous paper plus precipitation with phosphotungstic acid, and cadmium chloride.

‡ Preparation obtained by the multiple-precipitation process described in the previous section of this paper.

As may be seen from the last two lines of table 1, toxins purified by the methods other than alum and cadmium precipitation also show the same relationship of protein nitrogen to Lf units.

BACTERIAL PROTEIN AND TOXIN

As Bayne-Jones (1928), Hazen (1930) and others have pointed out, it may be demonstrated by appropriate immunological methods that the Ramon flocculation test is a true precipitation of diphtheria toxin by its specific antitoxin and not a precipitin reaction between bacterial antigens and antibodies. Under certain conditions the bacterial protein may form a precipitate with bacterial antibodies in the antitoxic sera thus obscuring the flocculation reaction. This occurred in our preparations with the green protein fractions which were adsorbed on the calcium phosphate precipitates. Locke and Main (1928) described similar bacterial proteins in their crude acid precipitates, which reacted with antitoxic sera in such a way that no optimum point of flocculation could be detected. These proteins were separated from the toxin by iso-electric-point fractionation.

In the method of purification described in this paper, most of the bacterial protein which interferes with the flocculation reaction is apparently removed by adsorption from the crude toxin on calcium phosphate, and precipitation at one-third saturation with ammonium sulphate. However, this does not insure that the purified toxins are entirely free of bacterial protein.

For the purpose of detecting bacterial protein in the purified toxins it was necessary to make an antibacterial serum practically free of flocculating antitoxin. Rabbits were immunized by repeated injections of 1.0 to 3.0 cc. of heavy suspensions of diphtheria bacilli of the Park 8 strain which had been killed by merthiolate and carefully washed. Subcutaneous injection of 0.5 cc. of the vaccine in a guinea pig caused local skin necrosis but had produced no other toxic symptoms after 21 days. The sera produced by the immunization of rabbits contained less than 1.0 A.T.U. per cubic centimeter, agglutinated the homologous bacilli strongly, and gave definite precipitin rings with crude toxin and various samples of bacterial protein but did not flocculate with toxin. Hazen (1930) has pointed out that agglutinins and precipitins for *Corynebacterium diphtheriae* are easily produced in rabbits, but flocculating antibodies are seldom obtained in these animals.

The results of bacterial precipitinogen titrations on dilutions of crude and purified toxins all done against the same lot of antibacterial serum are summarized in table 2. The third column gives the number of Lf units per cubic centimeter in the undiluted samples of toxin; the fourth column, the highest dilution of toxin solution which gave a definite ring with the antibacterial serum; and the last column, the number of Lf units per cubic centimeter in the toxin solution in this titer dilution. It may be seen from the last column of the table that there is no relationship between Lf units per cubic centimeter and titer

TABLE 2

The bacterial precipitinogen titer of various toxin preparations compared with the Lf-value

LOT NUMBER	METHOD OF PURIFICATION*	Lf UNITS PER CUBIC CENTIMETER	BACTERIAL PRECIPITINOGEN TITER	Lf UNITS PER CUBIC CENTIMETER AT TITER
	Crude toxin (old)	25	1:8	3.1
	Crude toxin (fresh)	14	1:64	0.22
	"Protein" fraction 1/3 saturated ammonium sulphate	6	1:16	0.43
	Green fraction adsorbed to calcium phosphate	7	1:16	0.38
2a	Method III, K	420	1:32	13.2
2e	III, K, Mg (a)	120	1:4	30.0
2e	Same, Mg (b)	23	1:2	11.5
2e	Same, Mg (c)	40	1:1	40.0
1a	III, Mg (a)	78	1:2	34.0
1a	Same, Mg (b)	120	1:3	40.0
1	III, Mg	160	1:5	32.0
F	III, Mg	420	1:32	13.2
M	(1)†	130	1:16	8.1
M	(2)†	125	1:8	15.6

* Explanation of symbols same as for table 1. Letters (a), (b), (c) after Mg indicate various fractions obtained by adsorption on colloidal magnesium hydroxide.

† (1) The preparation described in the section on multiple-precipitation process after precipitation of the crude toxin with acid and fractionation with ammonium sulphate.

(2) The same sample of toxin after completion of the multiple-precipitation process.

of bacterial precipitinogen. The figures vary from 0.22 Lf units per cubic centimeter at titer for one sample of crude toxin to 40 Lf units per cubic centimeter at titer for two samples of purified toxin, a difference of almost 200 fold. The purified toxins which contain the least bacterial protein per Lf unit are those from which certain impurities have been adsorbed by magnesium hydroxide.

Some of the precipitinogens obtained in extracts of *Corynebacterium diphtheriae* by Hazen (1930) were carbohydrates, but the precipitinogens under consideration in the present paper appear to be proteins precipitated or destroyed by heating for half an hour at 90°C. and pH 3.8 to 4.2.

The fact that the purified toxins give a positive, but relatively weak, test for bacterial protein may be due either to an immunological relationship of the toxin and bacterial protein, or to the presence of small proportions of bacterial protein mixed with toxin. As has been demonstrated by other investigators with crude toxin, it can be shown that the flocculation value of purified toxin is not at all affected after adding an excess of antibacterial rabbit serum, removing the resulting precipitate, and testing the mixture against antitoxin. This indicates that the bacterial precipitin tests and the flocculation reaction in purified toxins, as in crude toxins, are due to separate substances.

So far, attempts to completely separate the bacterial protein from the toxin by chemical methods applied to the highly purified preparations have been only partially successful. Such a separation has been accomplished by precipitating the toxin with nucleic or phosphoric acid at a pH of 5.0. Under these conditions most of the bacterial protein is left in the supernatant; but the flocculation time of the solutions of the precipitates dissolved in alkaline buffer is increased, and the toxicity considerably diminished.

CHEMICAL CHARACTERISTICS OF HIGHLY PURIFIED DIPHTHERIA TOXIN

From the results of tests for bacterial protein, coagulable and non-coagulable nitrogen, and the experiments on fractional ad-

sorption described in previous sections, the highly purified diphtheria toxins appear to consist of a mixture of proteins containing small amounts of proteose. No organic matter other than proteins and proteoses has so far been detected in these preparations. Although they do not consist of a single pure protein, the constancy of the Lf/protein N ratio in various purified preparations, and the results of experiments designed to separate the mixture of proteins indicate that the preparations contain one predominant protein, probably the toxin, mixed with smaller amounts of proteose and bacterial protein. The qualitative tests described below indicate that the predominant protein in these preparations has quite characteristic chemical properties.

In carrying out chemical tests on purified toxin preparations, it must be remembered that definite results can be obtained only with solutions containing at least 300 to 600 Lf units per cubic centimeter or a protein concentration of one to two parts per thousand.

The purified toxins coagulate when heated in concentrated solutions but solutions containing less than 100 Lf units per cubic centimeter may give no coagulum. Their behavior toward acid also varies with concentration of toxin and salt content. The proteins of most preparations are precipitated partially or not at all in the presence of one per cent sodium phosphate by acetic acid in the pH range 3.0 to 5.0. Trichlor-acetic and tannic acids precipitate 80 to 100 per cent of the protein. Ammonium sulphate and various metallic ions increase the precipitability of the protein by acid. The protein is salted out of solution between 0.4 and 0.7 saturation with ammonium sulphate.

The dried organic residue from the purified toxin preparations contains about 16 per cent of nitrogen. Tests for phosphorus were strongly positive on some of the less pure preparations (obtained by reagents free of phosphorus and phosphates), but were weak on the most highly purified toxins. The phosphorus content of these preparations was estimated to be less than 0.5 per cent. The phosphorus probably was present in an impurity. The purified toxins give consistently negative tests for sulphur

with lead acetate and with sodium nitroprusside even after prolonged boiling with concentrated sodium hydroxide.

A summary of the qualitative protein tests is given below. In all cases the tests were controlled by similar tests on horse serum diluted so as to contain about the same amount of protein as the toxin solutions tested.

Biuret—strongly positive, blue-violet.
Ninhydrin—positive but rather weak.
Millon—strongly positive; yellow changing to red.
Pauly diazo reaction—strongly positive; orange or yellow.
Sakaguchi test (arginine and guanidine)—strongly positive.
Hopkins-Cole—very weak.
Ehrlich, p-dimethylaminobenzaldehyde—very weak; light green.
Reichl test (tryptophane)—very weak; light green.
Molisch—weak or negative.

The chemical tests just described were done on purified toxins obtained by the alum cadmium-chloride method. A comparison of these results with the following summary of tests on the sample of concentrated toxin which had been purified by adsorption on aluminum hydroxide, precipitation with phosphotungstic acid, and precipitation with cadmium chloride, shows that toxins purified to the same degree by different methods are chemically similar.

Total nitrogen, 0.75 mgm. per cubic centimeter.
Milligram N per Lf unit, 0.0005; milligram N per M.L.D., 0.000075.
Dry weight combustible organic matter in solution 5.83 mgm. per cubic centimeter.
Content of nitrogen in organic matter, approximately 13 per cent.

Light yellow solution which gives heavy coagulum with heat and acid and dries down to light brown glassy residue. Biuret, strongly positive; lead acetate test for sulphur, negative; Molisch, very weak or negative; usual qualitative tests for copper and iron, negative.

Ash after ignition gives slight brown coloration with sodium sulphide, possibly due to traces of heavy metal.
No evidence of porphyrins or other significant colored compounds.

These tests indicate that the predominant protein in the purified toxin preparations has the usual protein linkages and contains among other amino acids considerable amounts of tyrosine, arginine and possibly phenyl alanine and histidine. The content of tryptophane is definitely very low as shown by the weak Hopkins-Cole, Reichl, and Erhlich tests, and the negative tests for sulphur indicate that cysteine is absent. Judging from the Molisch tests carbohydrates are absent.

The absence of sulphur and probably of tryptophane and the behavior toward acid and ammonium sulphate precipitation serve to characterize the protein in the purified toxins, and to differentiate it from many other commonly occurring proteins.

DISCUSSION

The most highly purified and active toxin preparations tend toward a uniformity in certain properties irrespective of the method of purification used. These constant properties are listed as follows:

1. Flocculation time, 4 to 6 minutes in solutions containing 100 Lf units per cubic centimeter, not over 40 minutes in solutions containing 20 Lf units per cubic centimeter.
2. Ratio M.L.D. per cubic centimeter/Lf units per cubic centimeter between 20 and 35.
3. Milligram nitrogen per Lf unit about 0.0005.
4. Not less than 80 per cent of the total nitrogen precipitable by trichlor-acetic acid.

Purified toxins which do not conform to these standards may be considered to have been altered during the process of purification, or are insufficiently purified. We have conducted no experiments with toxins prepared from media other than that described by Wadsworth and Wheeler (1934), but judging from the results of other investigators it seems probable that toxins prepared from other media would be found after adequate purification to have properties similar to those described here.

In the most active preparation so far obtained, the M.L.D. contained 0.000016 mgm. of nitrogen. This preparation contained about 16 per cent of nitrogen in the dried organic residue,

making the M.L.D. in terms of dry substance about 0.0001 mgm. for a 250-gram guinea pig, or 0.0004 mgm. per kilogram of guinea pig. This figure is very close to those for the lethal dose of purified ricin, 0.0005 mgm. per kilogram of rabbit, obtained by Osborne, Mendel and Harris (1905); and the M.L.D. of purified botulinus toxin, 10^{-8} grams for a mouse, obtained by Sommer, Sommer and Meyer (1928). This latter figure reduced to terms of toxicity per kilogram of mice comes out to be approximately 0.0005 mgm. Both the purified ricin and botulinus toxin, like diphtheria toxin, appear to be inseparably associated with proteins; but, unlike diphtheria toxin, they are not rapidly destroyed by digestion with proteolytic enzymes.

The purified toxic fraction behaves chemically as a protein, and the toxin is inseparable from protein by a number of different chemical procedures. The colored "porphyrin" compounds described by Coulter and Stone (1931, 1932) and Locke and Maine (1928, 1930) have been separated from the toxin during the course of the present work and we have found no evidence that the porphyrins bear any relation to the toxicity. We could obtain no definite evidence that copper, iron or other metals form an essential part of the toxin molecule.

It cannot be said that the diphtheria toxins purified by the methods described here are entirely free of impurities; but after repeated chemical precipitations the protein nitrogen per Lf unit appears to be approaching a limit of 0.0004 to 0.0005 mgm. Part of this protein nitrogen is undoubtedly bacterial protein, and most of the preparations contain in addition small proportions of non-protein nitrogen. Although the toxin probably forms a considerable percentage of the material in the protein portion, final purification must await the development of a method for separating these proteins in the purified toxin.

The presence of bacterial protein may slightly affect the flocculation of toxin by antitoxin, but the demonstrated relationship of Lf to M.L.D., indicates that the flocculating unit is largely a measure, in both crude and purified preparations, of toxin and toxoid, not of bacterial protein.

Like the crude toxin, purified diphtheria toxin has been ob-

served to produce in guinea pigs all of the characteristic lesions and symptoms. Skin necrosis and congestion of the adrenals occur frequently in animals injected with purified toxin. Also there are found petechial hemorrhages, congestion of the lymph nodes, ulceration in the gastro-intestinal tract, fluid in the peritoneum and pleura, changes in the heart, lungs and liver and late paralysis.

On the basis of present evidence, diphtheria toxin appears to be a single substance, probably a protein, containing an inseparable toxic group or property of unknown nature. Aging, formaldehyde and other chemical treatments produce a modification of the toxic factor itself without changes in other parts of the protein molecule. The loss of "avidity" described by Locke and Maine (1928, 1930) and others, as evidenced by impairment of flocculating and combining power, toxicity and antigenicity, might be explained as the result of denaturation of the whole protein molecule. Loss of "avidity" in toxin and denaturation of proteins are produced by the same chemical and physical agents.

These conceptions constitute, in our opinion, the best working hypothesis for use in further studies on the chemical nature of diphtheria toxin.

SUMMARY

With the progressive purification the ratio of nitrogen to Lf units in the toxin preparations appears to approach a constant value near 0.0005 mgm. N per Lf unit. None of the several purification processes so far used has yielded toxins with a lower N/Lf ratio.

The highly purified toxins consist mainly of a protein which is not easily precipitated with acid at any pH and contains no cysteine sulphur and very little or no tryptophane. Traces of proteoses from the culture medium, and of other proteins probably derived from the bacterial cells are also present.

The chemical, immunological and physiological properties of diphtheria toxin observed in the course of this work indicate that it is a single protein-like substance.

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MICROCOCCUS TETRAGENUS INFECTION

II. DESCRIPTION OF VARIANT FORMS[1]

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About 40 years ago a controversy existed as to whether numerous strains of *Micrococcus tetragenus* existed or if the various kinds then studied were merely variant forms of a single strain. Those favoring the first point of view denoted separate strains by the terms, *subflavus* (Von Besser), *aureus, albus* (Boutron), *citreus* (Vincenzi) and *ruber* (Bujwid, Trémolieres). Others believed these to be merely variant forms of a single strain. It appears now, as will be shown in this report, that both views were correct; numerous strains exist and each has variant forms.

Among the early investigators were Boschi and Bellei (1897) who noted that a yellow variety lost its chromogenic ability and became white on subsequent transfer and believed that one was a variant form of the other. Similar views were held by Jacobelli (1899) and Vincenzi (1897). Teissier (1896) in 1896 noted that the typical tetrad formation which occurred *in vivo* became less and less marked on subculture *in vitro*. He also observed the growth of pinpoint daughter colonies in the original large colonies and the development of a collarette around the mother colony. In 1914 Eisenberg noted the appearance of two distinct colony forms, a large form composed of capsulated virulent tetrads and a small form composed of noncapsulated cocci. Baerthlein in 1918 also noted the appearance of small brownish-yellow, delicate colonies among the typical large yellow ones. The large colonies were composed of typical tetrads, the small of small cocci. Wreschner in 1921 isolated a large gray-white virulent capsulated

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TABLE 1

Biologic characteristics of the variant forms of a strain of M. tetragenus isolated from a patient (Reimann, 1935)

COLONY FORM ON AGAR	COCCUS FORM	GROWTH IN BROTH	GELATIN LIQUEFIED	ACID IN						HEMOLYSIS	PATHOGENIC FOR MICE	GROWTH IN						RESISTANT TO			
				Glucose	Sucrose	Maltose	Lactose	Mannitol	Milk			10°C.	41°C.	pH 5.5	pH9	CO_2	10 per cent salt agar	Ultraviolet light, 1 minute	Freezing 45 times	56°C. for 15 minutes	0.4 per cent formalin, 15 minutes
Mucoid-yellow—thick, round, translucent, mucoid, coherent. Unstable	Small cocci in tetrads. Capsulated	Granular yellow sediment mucinous	−	−	−	−	−	−	−	−	−	−		−	+	+	−				
Yellow—thick, round opaque, buttery. Stable	Large and small cocci in tetrads, no capsule	Granular yellow sediment	+ in 3 wks.	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	+	+	+
White—thick, round, opaque, buttery. Greenish on blood agar. Stable	Small cocci, single, diploform tetrads or groups. No capsule	Granular sediment. Spirals on shaking	−	±	±	±	±	−	−	−	−	−	+	+	−	+	+	−	−	−	−
Small white—smaller than the white. Unstable	Same as above	Granular sediment. Spirals on shaking	Variable																		
Translucent—thin, occasionally autolytic, yellow-blue, iridescent. Hemolytic	Pleomorphic cocci, minute to large in size. In groups seldom in tetrads	Diffuse with sediment on shaking	+ in 2 days	+	+	+	−	−	−	+	−	−	+	+	−	+	+	−	−	−	−

| | | |

form from the sputum, which in subculture lost its capsule and virulence and grew as a small white flat colony. The small form would occasionally revert to the large on animal passage. No specific serum could be prepared from the use of capsulated forms. The decapsulated form was clumped in a dilution of 1:1600 by its homologous antiserum. The mode of division of the cocci was carefully studied by Bosc and Galavielle (1899).

In a previous paper (Reimann, 1935) the clinical features of *M. tetragenus* infection were reviewed and a case was described, characterized by septicemia with purulent arthritis and meningitis, in which this organism was isolated from the blood, joint fluid and spinal fluid. The morphologic characteristics of the cocci seen in pus from the knee and the development of white porcelain-like colonies from the blood and spinal fluid suggested that the organisms existed in the "white" form in the body. When the culture plates were aged, evidence of microbic dissociation appeared. Numbers of the white colonies turned yellow or developed yellow daughter colonies and on several occasions a translucent variety appeared. The three forms reproduced their own kind on repeated sub-culture, but when the plates were incubated for long periods transformation of one form into another was often observed. The three forms were characterized by different and distinctive biological properties which are summarized in table 1.

The present paper embodies a detailed description of these 3 variant forms and of several other variant forms induced to appear by experimental methods. Subsequent papers will deal with immunologic aspects, the nature of the pigments elaborated by the chromogenic variants, and the resistance of the variant forms to unfavorable environments.

METHODS

Colony growth and morphology were observed in ordinary petri plates filled to a depth of 5 mm. with nutrient agar; for point-colony observations the agar was 10 mm. thick. The plates were inoculated, sealed with adhesive tape and placed at 37°C. for 18 hours, then at room temperature (±25°C.). The higher

temperature hastened growth, but drying was less rapid at 25°C. Point-colony plates were seeded by touching the agar at 2 or 3 widely separated points with the tip of a wire which had been immersed in a dilute suspension of culture. Agar foci were prepared according to the technique described previously (Reimann, 1927).

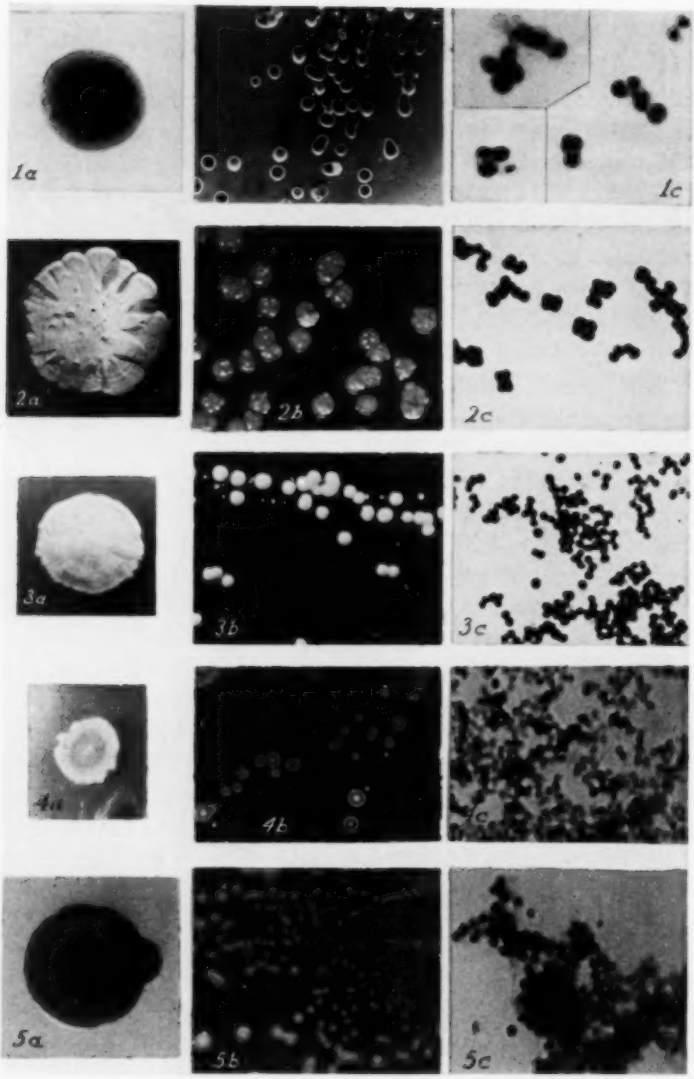
DESCRIPTION OF THE VARIANT FORMS

The white colonies

When the spinal fluid, pus from the knee or blood were plated, large white colonies grew slowly. After 3 or 4 days the colonies measured 2 to 4 mm. in diameter and became flat with a porcelain-like luster (figs. 3a and 3b). When left at 25°C. for a month or more, the colonies either ceased to grow and remained unchanged or (*a*) gradually turned yellow as a whole, (*b*) developed a yellow or brownish sector with the apex toward the center, (*c*) developed daughter white colonies or (*d*) developed numerous daughter colonies which were bright yellow, like amber beads. These daughter colonies would frequently appear rather suddenly within 24 or 48 hours. On one occasion, white colonies, plated on blood-agar gradually assumed a brownish or buff color after 6 weeks. Plain agar slants covered with a growth of white colonies, when stored in the ice-box at 10°C., frequently developed numerous bead-like daughter yellow colonies after a month or two.

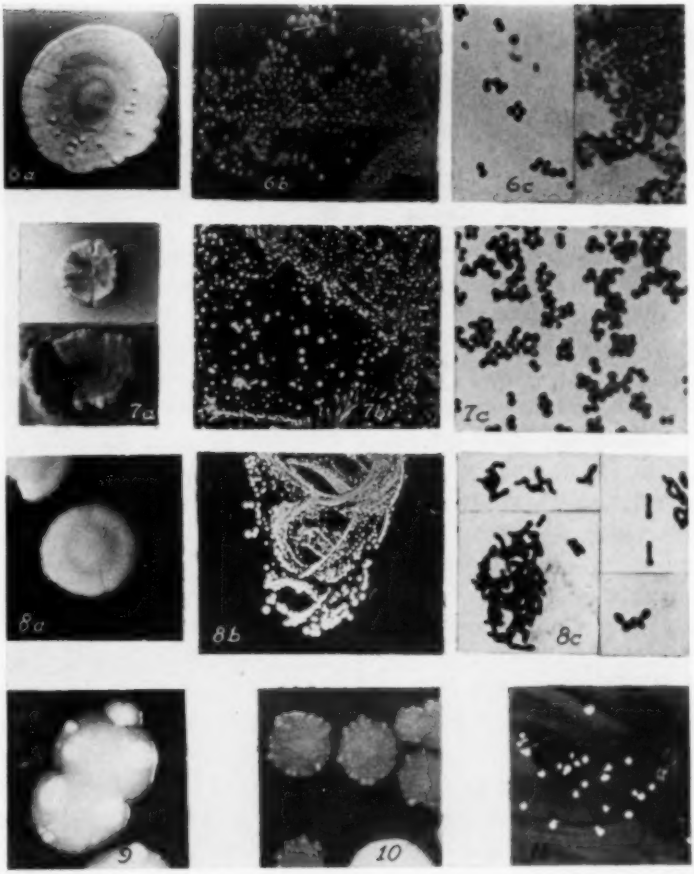
Subcultures from the white colonies usually reproduced their own kind, but when conditions were not optimal numerous minute white colonies often appeared among the larger ones (fig. 3b). These minute colonies never attained the size of the large ones. Transfers from the large white forms reproduced only the large white; transfers from the small colonies usually reproduced both small and large colonies. On some occasions, on plates seeded with small colony cocci, translucent colonies of entirely different characteristics were found, at others, yellow colonies developed. It appeared as if the small white colonies were less stable than the large ones and served as a transitional stage toward other variant forms.

Agar plates 10 mm. deep were inoculated with a needle at one



FIGS 1 TO 8

Figures 1a to 8a are natural size photographs of point colonies grown at 25°C. for two to four weeks of the following variant forms: 1a, mucoid-yellow; 2a, yellow; 3a, white; 4a, translucent; 5a, mucoid-pink; 6a, pink; 7a, brown, and 8a, bacillary form. Figures 1b to 8b are photographs of colonies of the respective variants two to five days old growing on agar plates at 25°C. (slightly enlarged). Figures 1c to 8c are photographs of the respective organisms stained by Gram's method (× 1500). The mucoid-yellow (1c) are enlarged 4300 ×. Photographs of the yellow-pink variant appear like those of the white form and are not shown



here. The mucoid-white resembled the mucoid-pink when photographed and is also not shown here. Note especially the stellate portion of yellow in the mucoid-yellow colony (1a) as shown by transmitted light. In figure 1b are seen two forms of colonies, mucoid-yellow and yellow, some joined and some separated. Capsules are shown in figure 1c. In figure 3b, both large and small white colonies are shown. The mucoid-pink colony in figure 5a was tipped to the right before photographing to show the fluidity of the colony as it formed the protuberance at the right. The capsules of the mucoid-pink forms are visible in figure 5c. Two brown colonies are shown in figure 7a, the upper with alternate white segments, the lower with a large wedge of yellow.

FIG. 9. ORIGINALLY YELLOW COLONIES ENLARGED 5 ×

After four weeks at 25°C. a halo of white forms developed. On the original central portion are daughter yellow colonies, on the halo are daughter white colonies.

FIG. 10. TRANSLUCENT COLONIES SIX DAYS OLD STUDDED WITH WHITE DAUGHTER COLONIES (× 5)

FIG. 11. PORTION OF A PLATE SEEDED WITH PINK FORMS ONE MONTH OLD

White colonies composed of bacillary forms appear among the pink forms

point only with cocci from a white colony. Under these circumstances the colony, unhampered by neighbors, grew to giant size in several weeks often becoming 10 or 15 mm. in diameter and 1 mm. thick. The surface was porcelain-like and marked with radial and concentric ridges (see fig. 3a). On the eighteenth day of growth one colony developed a buff-brown colored sector. One 5-day-old point colony was 8 mm. in diameter. It appeared to lose its opacity slowly and on the eighth day two delicate white wedges appeared on opposite sides of the now translucent colony. The wedges gradually became larger and united, (more or less resembling an hour glass dividing the original portion of the colony and later extending delta-like beyond it). The new growth became more and more opaque and finally resembled the original white form. Subcultures from it produced typical large white colonies. The original portion of the colony became transparent and after 30 days was almost invisible. In its place numerous minute white daughter forms were left composed of clusters of minute cocci which on subculture produced translucent colonies. After 44 days the central areas of the wedge shaped growth became sulfur-yellow while the periphery remained white and was studded with daughter white colonies. It was thus possible to observe in a single colony variation from the white to the translucent, to the white and finally to the yellow form. A flask culture was allowed to remain at 25°C. for 5 months. Plates made from this culture after 3 days' incubation showed colonies which were small and translucent; 3 days later all the colonies turned brown.

Growth in homologous immune serum. No variation was noted on plates seeded from each transfer of a culture through a series of 12 tubes containing 10 per cent of homologous immune serum in broth at 3-day intervals.

Passage in lithium chloride broth. The white organisms were transferred at 48-hour intervals at 37°C. through 29 tubes of broth containing 1 per cent lithium chloride. No evidence of dissociation occurred on plating on agar until the fifth transfer when, among hundreds of white colonies, there appeared 1 yellow colony and a few translucent colonies. Further passage did not

induce any greater dissociation. At the fifteenth, twenty-third and twenty-eighth transfers similar variants were noted. No further variation occurred even after plates were made from the same tubes which were left at 37°C. for 3 months.

Dissociation in vivo. Melted agar at 42°C. was mixed with a heavy suspension of white organisms and 1 cc. was inoculated subcutaneously in mice. The lump formed by the agar was aspirated and the material plated on agar after 7 and 14 days. Numerous cocci, cell detritus and leucocytes were seen in smears but phagocyted cocci were rarely seen. The organisms were often pleomorphic and some giant in size. The cultures grew slowly as did the ones from the patient when first cultures were made on agar. Some colonies were still invisible to the unaided eye after 4 days' incubation. Most of the colonies remained white, but in a few, yellow wedges appeared; in others the center became yellow and on some, yellow daughter colonies developed. Numerous translucent colonies appeared among the white ones. In one mouse killed after 21 days no organisms could be cultivated from the site of the agar focus, nor from other organs.

The white organisms. The colonies emulsified with ease and the cocci remained in diffuse suspension. The individual organisms were usually uniform in size, grouped in clusters like staphylococci, or singly, in pairs and occasionally in tetrads (fig. 3c). They exhibited varied staining reaction with Gram's technic. On the same slide, cocci in some areas would be found deeply Gram-positive, in others entirely decolorized, with various intermediate gradations. No capsule could be demonstrated. Growth in broth was often finely granular until shaken. In cultures more than 24 hours old a gummy deposit formed which when twirled formed a spiral filament. Other biological characteristics are listed in table 1.

The yellow colonies

The yellow colonies grew more luxuriantly and rapidly and were thicker than the white forms. When very young and crowded the colonies were creamy white but soon became pigmented sulfur- or lemon-yellow, especially where isolated. They

appeared to be the most stable of all forms, reproducing their own kind under many adverse conditions. Upon ageing a plate on which the colonies were somewhat crowded, several changes occurred. Some colonies developed yellow daughter colonies or heavier yellow sectors (fig. 2b). Around some colonies a white halo developed on which white daughter colonies appeared (see fig. 9). Subcultures from the yellow portion produced yellow forms, and from the white halo, white forms grew.

Point colonies of the yellow form grew to giant size, often to 22 mm. in diameter and 2 mm. thick. The colonies when young were round and smooth, later they became heaped-up, buttery and crinkled (fig. 2a). Daughter yellow colonies occasionally appeared. Wedges of mucoid yellow or of white occasionally appeared. On one occasion the wedges of white increased until the whole colony became white.

Growth in homologous immune serum. The yellow form was transferred fourteen times at approximately 3-day intervals in 10 per cent anti-yellow serum. Plates were made from each tube after the last passage. The plate from the first passage showed mostly yellow colonies with 3 or 4 small white ones. The second passage plate showed mostly yellow colonies with numerous translucent ones many of which turned yellow in 2 or 3 days. The proportion of translucent to yellow increased during the third and fourth transfers, then diminished until, after the sixth or seventh transfers, no more were seen among the usual yellow colonies.

Dissociation in vivo. Material was aspirated from agar-foci containing yellow organisms embedded in mice at 7 and 14 days. Stained smears revealed numerous large tetrads, detritus, pus cells with but little evidence of phagocytosis. Cultures grew slowly, usually produced a majority of yellow colonies but a few white ones occasionally appeared which gradually turned yellow. Organisms were no longer recovered after the twentieth day *in vivo*.

The yellow organisms. Cocci composing the yellow colonies were usually grouped in characteristic large tetrads. There were often two distinct kinds present, large and small, each grouped

in fours (fig. 2c). No definite capsules were seen. Growth in broth was often flaky and a gummy mass settled out which formed spirals when twirled. Gelatin was slightly liquefied after 3 weeks at 25°C. It was suspected that reversion to the translucent form might have caused the liquefaction, but a plate made from the gelatin tube showed yellow forms alone. The yellow variety failed to ferment sugars (table 1), but caused softening of Dorset's egg medium, softening and clearing of coagulated blood serum and occasional softening and erosion of agar.

Mucoid-yellow form

A flask-broth-culture of the yellow form was allowed to remain at 25°C. for 3 months. The bouillon became mucinous and yellow. A plate made at the end of this time revealed a rich growth of translucent, mucoid, confluent colonies which when touched were glairy and coherent (fig. 1a and 1b). After a day or two the colonies took on a yellowish cast and resembled translucent yellow glass. Point yellow colonies occasionally developed similar mucinous translucent yellow wedges. In biological tests involving growth at low and high temperatures, gelatin liquefaction, hemolysis and sugar fermentation the organisms behaved like those of the original yellow form. After several days on agar some of the colonies gradually became drier, friable, opaque and yellow or developed yellow opaque daughter colonies like the original form (fig. 1b). On another occasion the mucoid-yellow and opaque-yellow forms were plated side by side. Each form reproduced its own kind for 3 passages, after which the mucoid form became opaque. The mucoid-yellow on the first and second transfer plates also became opaque after several days. On the side seeded with the mucoid-yellow form, numerous minute translucent colonies also appeared. These when subcultured produced both mucoid-yellow and minute translucent colonies. Cocci from the small translucent ones were hemolytic. Point colonies of the mucoid-yellow variety after a week at 25°C. were 8 mm. in diameter and mucinous. As the colony aged the central portion often became opaque which later spread stellatewise, peripherally, until the whole colony became opaque (fig. 1a).

Cocci from the mucoid-yellow colonies were usually smaller than those of the original yellow colony but were still arranged in groups of four. In certain portions of a stained preparation on a slide no capsule was demonstrable, but on others a definite pink-stained area surrounded the tetrads (fig. 1c). The capsules although plainly visible were difficult to photograph. The best results were obtained by using an orange filter and Wratten M plates. There was much pink stained amorphous material present which occasionally appeared to be composed of decolorized and degenerated cocci. When the organisms were injected subcutaneously into mice in agar foci, no evidence of reversion to other forms were observed. The organisms did not survive long and were no longer cultivable after 2 weeks *in vivo*.

The translucent colonies

The translucent form originated chiefly from the small white colonies. The colonies were delicate, of a yellow-blue iridescence and permitted type to be read through them (figs. 4a and 4b). They often adhered to the medium but could be easily emulsified in broth. On blood-agar large areas of hemolysis surrounded the colonies. After repeated passages at 4-day intervals on plain agar, the hemolytic power disappeared and the colonies became more and more opaque like the white forms. During the process of transferring, occasional large yellow or white colonies developed on the plates, indicating a reversion or transformation of the translucent forms to the white and yellow forms. At the eighteenth transfer the colonies were all white but when plated next to the original white form, a difference was still evident.

During lithium-passage experiments on the white form, several translucent colonies appeared at the twenty-eighth passage. Organisms from a translucent colony were inoculated on another plate. There appeared a mixture of white and translucent colonies. After 6 days at 25°C. the translucent colonies grew to about 2 mm. in diameter. On their surfaces appeared numerous whitish daughter colonies (fig. 10). The original mother colony then gradually disappeared until only a sprinkling of the daughter

colonies remained. Cocci from these colonies behaved like those of the white forms. A flask culture 5 months old when plated revealed only translucent forms. The large colonies developed brownish rather opaque centers and a sharply demarked translucent rim.

Growth in homologous immune serum. During the first 3 transfers in 10 per cent homologous immune serum, an occasional white or yellow colony appeared among the translucent forms. On a plate made from the fourth passage tube numerous yellow, white, pink and brown colonies appeared among the translucent forms. Thereafter no important changes occurred after fourteen transfers.

Point colonies grew poorly, seldom exceeding 5 or 6 mm. in diameter (fig. 4a). The margins were usually irregular and the center became autolyzed and transparent.

Agar focus experiments. A subcutaneous agar mass inoculated with the translucent forms was aspirated 7 days after injection. On plating the material two forms of colonies appeared, one rather heavy but still translucent; the other very thin and delicate which became fainter until after 3 weeks it was no longer visible. Daughter colonies occasionally appeared on these "suicide" colonies and remained after the original colony had disappeared. Transplants from these colonies reproduced translucent colonies, but an occasional large yellow form or white form appeared.

On another occasion material from an agar focus when planted produced about 20 translucent colonies after 18 hours at 37°C. After 48 hours at 25°C. about 50 per cent of the colonies appeared to be distinctly thicker resembling the white form. One week later several of the colonies turned bright orange-buff or brownish in color, and a few days later others gradually assumed a brilliant shell-pink color. Two or 3 yellow forms were also present. There were therefore at one time on the same plate, translucent, white, yellow, pink and brown colonies.

The translucent cocci. Organisms from the translucent colony were distinctly different from those of the other variant forms. They were minute cocci, usually retained the Gram stain, were markedly pleomorphic and many of the cocci were just within the

limit of vision with the oil immersion system; yet they failed to pass through a Berkfeld Vn. filter. They were usually arranged in irregular clusters (fig. 4c). In certain stained films much Gram-negative detritus apparently composed of autolyzed cocci was present. The cocci were hemolytic, liquefied gelatin in a few days and formed acid in certain sugars (table 1).

The pink colonies

These forms appeared to originate from the translucent variety, but an occasional pink form was observed among the white colonies on a 3-weeks-old agar plate made from a strain of *M. tetragenus* derived from another case of meningitis. Except for the pigment the colonies resembled those of the white form (figs. 6a and 6b). They reproduced themselves when transferred after more than 70 passages on agar, but white or yellow variants occasionally appeared on the plates. The pigment varied in shade from a delicate shell-pink to a deep coral and was especially brilliant when plates were incubated at 10°C. A flask culture 2 months old when plated revealed pink and translucent colonies. A similar flask culture of a pink variant from a different strain (Arens) when plated showed a predominance of pink colonies with several white, yellow, brown and translucent colonies.

Point colonies attained a diameter of 12 to 22 mm. after 17 days at 25°C. The colonies were flat cones, 1 mm. deep at the center and marked with radial and concentric striae (fig. 6a). The center was often more deeply pigmented. Some colonies after 3 or 4 weeks developed white wedges composed of white forms, and others formed yellow wedges, further illustrating reversion to the white and yellow forms. A new variant appeared on one occasion. A yellowish wedge appeared in a point colony 3 weeks old. After 6 weeks when growth had nearly ceased the colony was surrounded by a delicate fringe-like growth of similar color. Cocci from the yellowish wedge produced yellowish-pink colonies which are described later.

Variation in vivo. An agar focus inoculated with pink cocci was aspirated on the seventh and tenth days and plated. Before the third day all the resultant colonies were translucent. After

this time about one-half of the colonies gradually became opaque and pink. Two large yellow colonies appeared later and many of the original translucent colonies disappeared by autolysis. Curiously, when the same aspirated material was first incubated in broth before plating, the resultant colonies were all translucent and remained so without evidence of dissociation.

The focus was again aspirated after 17 days but only one large yellow and a few translucent colonies grew. None grew from material aspirated on the twenty-first day.

Cocci from the pink colony. The cocci usually resembled those of the translucent form in smallness and pleomorphism, but occasionally were more like those of the white forms (fig. 6c). The biological characteristics were similar to those of the white forms. The pink form grew poorly in broth especially so in the presence of serum. No growth occurred in 10 per cent serum. During attempts to transfer this form in homologous immune serum it was necessary to begin with a 1 per cent dilution. Even under these circumstances growth occurred only after 4 or 5 days. Growth on agar plates containing 5 or 10 per cent "anti-pink" serum or normal serum was correspondingly slow. No reversion to other forms occurred.

Mucoid-pink colonies

A flask culture of the pink form 1½ months old when plated showed mostly mucoid or watery pink colonies with a sprinkling of the usual opaque pink and translucent colonies, many of the latter being autolytic. On one occasion a mucoid-pink colony developed alongside a mucoid-yellow colony on a plate seeded with the latter form. The translucent pink and opaque pink reproduced their own kind after several transfers. On agar plates there was an apparent tendency for the mucoid-pink to revert to the pink form. Point colonies resembled drops of thin pink mucin which would run when the plate was tipped (fig. 5a). On several month-old mucoid-pink point colonies there appeared a white wedge. Subcultures from the white wedge produced large mucinous mucoid-white colonies.

Cocci from the mucoid or watery pink colonies were highly

limit of vision with the oil immersion system; yet they failed to pass through a Berkfeld Vn. filter. They were usually arranged in irregular clusters (fig. 4c). In certain stained films much Gram-negative detritus apparently composed of autolyzed cocci was present. The cocci were hemolytic, liquefied gelatin in a few days and formed acid in certain sugars (table 1).

The pink colonies

These forms appeared to originate from the translucent variety, but an occasional pink form was observed among the white colonies on a 3-weeks-old agar plate made from a strain of *M. tetragenus* derived from another case of meningitis. Except for the pigment the colonies resembled those of the white form (figs. 6a and 6b). They reproduced themselves when transferred after more than 70 passages on agar, but white or yellow variants occasionally appeared on the plates. The pigment varied in shade from a delicate shell-pink to a deep coral and was especially brilliant when plates were incubated at 10°C. A flask culture 2 months old when plated revealed pink and translucent colonies. A similar flask culture of a pink variant from a different strain (Arens) when plated showed a predominance of pink colonies with several white, yellow, brown and translucent colonies.

Point colonies attained a diameter of 12 to 22 mm. after 17 days at 25°C. The colonies were flat cones, 1 mm. deep at the center and marked with radial and concentric striae (fig. 6a). The center was often more deeply pigmented. Some colonies after 3 or 4 weeks developed white wedges composed of white forms, and others formed yellow wedges, further illustrating reversion to the white and yellow forms. A new variant appeared on one occasion. A yellowish wedge appeared in a point colony 3 weeks old. After 6 weeks when growth had nearly ceased the colony was surrounded by a delicate fringe-like growth of similar color. Cocci from the yellowish wedge produced yellowish-pink colonies which are described later.

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Cocci from the mucoid or watery pink colonies were highly

pleomorphic and stained smears showed much amorphous material. The cocci were occasionally surrounded by pink-staining irregular capsule-like substance (fig. 5c). No evidence of reversion occurred when the organisms were inoculated into agar and allowed to remain in mice. Cocci which produced mucoid-pink colonies were recoverable after 2 weeks *in vivo*. Cocci from the mucoid-pink colony were specifically agglutinated by antiserum prepared with cocci from the mucoid-yellow colonies. The mucoid-pink pigment was different from that of the pink colonies.

Mucoid-white colonies

Colonies of this form were first encountered after a year of observation. They were noted as wedges which appeared in certain mucoid-pink colonies. Mucoid-white colonies also appeared on agar plates seeded from an 8 month-old broth culture of the white form. Except for the pigment, both colony and cocci resembled those of the mucoid-pink form. Point colonies grew well occasionally turning pinkish as if reverting to the mucoid-pink, and in old cultures opaque white daughter colonies appeared. The cocci varied in size and were often surrounded by a pink-stained capsule-like substance. Cultures made from agar foci containing the mucoid-white forms in mice showed that the organisms died off rapidly without evidence of dissociation. The cocci were not virulent for mice.

The pink-yellow colonies

This form obtained from a sector in a point pink colony bred true on subsequent culture, forming colonies resembling the pink form except for the pinkish-yellow color. The biologic behavior of the cocci also resembled that of the pink form. Point colonies usually showed concentric rings of different shades, the center pinkish-yellow, surrounded by a distinct pink area in turn surrounded by a pinkish-yellow rim. As will be shown in a later publication the color was due to the presence of at least two pigments. When grown at temperatures over 25°C., the yellow pigment predominated but at 10°C., the colonies became deep coral-pink. The cocci agglutinated specifically in homolo-

gous antiserum and in antiserum made with the yellow and the pink forms.

Bacillary form

On numerous occasions white colonies of bacilli were noted among the pink forms, but were regarded as contaminants. Later, their constant appearance in cultures derived from repeated single colony cultures of the pink form led to closer scrutiny. On numbers of plates seeded with the pink form there appeared the usual pink colonies. After ageing for about a month at 25°C., whitish daughter colonies appeared among the pink (fig. 11) which were first believed to be composed of cocci of the white form, but examination revealed them to be composed of short, pleomorphic, metachromatic bacilli (fig. 8c). The whitish colonies usually grew several days or weeks after pink forms had appeared, pushing up from beneath or appearing in and among the pink colonies. They were never encountered in uninoculated portions of the plate or among colonies of the other variant forms. Bacillary forms were occasionally encountered in old broth cultures of the pink variant and in cultures of pink variants of two strains from other sources.

Point colonies at 25°C. grew slowly and formed dome-shaped, shiny, opaque white colonies (fig. 8a). On ageing an irregular thin outgrowth studded with daughter colonies appeared. Reversions to the coccus forms were not observed on ageing or during residence in agar foci in mice. The bacilli were often regular in size, shape and staining. Occasionally all were Gram-positive, or all were Gram-negative. Usually toward the center of slide preparations they were Gram-negative and toward the edges Gram-positive. Occasionally Gram-positive and Gram-negative rods were present in the same field. Pleomorphism occurred and was especially marked in broth cultures. Rods with one end or both ends pear-shaped or bulbous, like a drum-stick or a dumb-bell were seen; some had a globular enlargement in the center and others were beaded. The bulbous ends frequently appeared as if separating from the rod portions suggesting their budding off or breaking off as cocci (fig. 8c).

Reversion to the coccus forms such as was reported by Schmidt-Kehl (1930) was not observed.

The brown colonies

The brown colonies resembled those of the white form but for the pigment. The pigment was less intense than that of the pink or yellow varieties and varied from buff to orange-buff to tan or brown. Pigment was often invisible where colonies were crowded and was best developed in discrete colonies. Usually plates revealed colonies of varying shades from whitish to brown. The colonies reproduced themselves without much evidence of variation for more than twenty transfers in agar, except for the occasional appearance of a white or yellow form.

Point colonies were not as luxuriant as those of other forms but grew better than those of the translucent variety. They were usually thin and flat with a raised somewhat wavy margin. Most of the point colonies were porcelain-white and developed irregular sectors and patches of orange-brown or buff. Often the apex of the triangular areas of pigment was pointed toward the periphery. One colony was composed of alternate sectors of yellow and brown (fig. 7a).

Agar focus experiments. Agar foci inoculated with brown cocci were aspirated and plated after 10 and 17 days. At both times the colonies at first were all white but later most of them, especially when isolated, became brownish. After a few days the pigmented ones became deeper colored and more of the white ones became brown colored so that all gradations from white to rich orange-brown were present. Occasionally the whitish colonies developed marginal brown daughter colonies, some developed an orange-buff rim while others were brown with a white rim. One large yellow colony appeared. It appeared that the brown variety dissociated more easily after ageing on agar than *in vivo*, in contrast to the pink form in which the opposite was noted.

Cocci from the brown colonies were metachromatic, regular in size and were arranged in clusters and occasionally in fours like

those of the white variant (fig. 7c). The biological reactions were like those of the white form (table 1).

Dissociation of M. tetragenus from other sources. Five strains were obtained from Pribram's culture collection in Vienna, labelled and received as follows: *albus*, creamy white colonies; *ruber* (Bujwid), translucent confluent colonies with pink daughter colonies; *Henrici*, sulfur yellow colonies; *citreus*, white colonies; *Schnürer*, white colonies; one strain was received from the American type culture collection labelled *159*, and a number were obtained from local sources, as follows, *Perkins* from a case of meningitis, and 6 others from sputa. All of the strains corresponded in general to the usual criteria laid down for *M. tetragenus*. There was constant evidence of reversion chiefly from the white to the yellow or *vice versa*, in most of the strains when colonies were aged on agar plates. Two strains, *159* and *Albus* were especially resistant to dissociation.

When these strains were mixed with agar and injected subcutaneously into mice, most of the variant forms were derived from all but two. Of special interest was the strain *ruber*. It appears to represent the pink variant form of an original yellow or white and is in reality not a separate strain. The stability of this variant form is indicated by its propagation as such in a culture collection for more than 42 years. The variant forms of these strains behaved in most respects like the strain studied in detail as regards coccus morphology, gelatin liquefaction, hemolysis, sugar fermentation and variation.

Virulence. Although many mice, rabbits and guinea pigs were inoculated intraperitoneally, subcutaneously and intravenously with *M. tetragenus* from each strain studied in all its variant forms, none succumbed to infection. Agar foci in mice and rabbits inoculated with each of the variant forms contained viable cocci for as long as 3 weeks without evidence of illness. In all of the agar foci, the cocci seemed gradually to disappear, apparently from inability to grow in an unfavorable environment without evidence of active phagocytosis. Addition of mucin to the inoculum was without effect on the virulence. Rabbits were

immunized by intravenous injection of large doses of living cocci without ill effects. Rapid transfer on media failed to enhance virulence. Mice sometimes died after a massive dose but subcultures were if anything even less virulent.

It is difficult to reconcile these observations with the statement of many investigators who claim *M. tetragenus* to be highly pathogenic for white mice, and use this behavior as a point of differentiation from the staphylococcus. Furthermore, the fact that *M. tetragenus* is a common inhabitant of the nasopharynx would presuppose its frequent appearance in mice inoculated with sputa for the purpose of typing pneumococci, granting its pathogenicity for these animals. I have never encountered *M. tetragenus* in using the mouse method of typing hundreds of sputa, nor has it been encountered by other observers of wide experience.[2] That strains virulent for mice do exist is indicated by the recent studies of Stübinger (1934) and by those of many other observers cited in table 2 of a previous paper (Reimann, 1935).

Through the courtesy of Professor Kisskalt of Munich, 2 strains of *M. tetragenus* were obtained labeled "Lodi" and "J. F. I." which were said to be virulent. Intraperitoneal inoculation of heavy suspensions of these strains occasionally caused the death of white mice after 2 weeks. The cocci obtained from the peritoneal cavity and blood of the mice were capsulated and produced large mucoid, white colonies. Studies of these strains are in progress.

SUMMARY

A strain of *Micrococcus tetragenus* isolated from a patient was subjected to various experimental procedures known to favor microbic dissociation. Ageing on agar or in broth and residence *in vivo* were most effective. As a result 11 variant forms were encountered and studied, namely, mucoid-yellow, yellow, mucoid-white, white, small-white, translucent, mucoid-pink, pink, pink-yellow, brown and a bacillary form. The phenomena observed are discussed in study III.

[2] Personal communications to the author from Drs. Avery, Cooper, Beckler and Finland.

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MICROCOCCUS TETRAGENUS INFECTION

III. IMMUNOLOGIC STUDIES OF VARIANT FORMS, AND DISCUSSION[1]

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Immune sera were prepared by using mucoid-yellow, yellow, white, translucent, mucoid-pink, pink, pink-yellow, brown and bacillary forms of *Micrococcus tetragenus* as antigens, for the purpose of studying the immunological relationships of these variant forms. (Reimann, 1936)

METHODS

Immune sera were prepared in the usual manner by inoculating rabbits intravenously with heavy suspensions of living organisms of the respective dissociants daily for 5 days and repeating the course twice more, after alternate rest periods of similar duration. Agglutination tests were performed in the usual macroscopic manner. Suitable suspensions of organisms were added to appropriate dilutions of immune serum in normal physiological salt solution as indicated in tables 1 and 2. The tubes were incubated at 37°C. for 2 hours, placed in the ice-box over-night and read.

Autolysates were obtained by incubating flask cultures of 100 cc. of broth for 2 months or more at 25°C. The broth culture was then centrifuged and the clear supernatant fluid was used for the tests. In the case of the mucoid-yellow and mucoid-pink cultures it was necessary to filter the viscid broth through a Berkefeld candle to obtain a clear fluid.

Precipitin tests were performed by adding 0.5 cc. of ascending dilutions of clear supernatant fluid to tubes containing 0.3 cc.

[1] Aided by a grant from the University of Minnesota Graduate School Fund.

TABLE 1

Agglutination reactions of variant forms in immune serum prepared with these forms

			DILUTION OF SERUM						
			1:10	1:20	1:40	1:80	1:160	1:320	1:640
Anti-mucoid yellow serum		Mu-Y	+++	+++	+++	+++	+	−	−
		Y	−	−	−	−	−	−	−
		W	−	−	−	−	−	−	−
		T	−	−	−	−	−	−	−
	Cocci	Mu-P	+++	+++	+	−	−	−	−
		P	−	−	−	−	−	−	−
		P-Y	−	−	−	−	−	−	−
		B	−	−	−	−	−	−	−
		Bacilli	−	−	−	−	−	−	−
Anti-yellow serum		Mu-Y	+	+	−	−	−	−	−
		Y	−	+	+	+	+	−	−
		W	−	−	−	−	−	−	−
		T	−	−	−	−	−	−	−
	Cocci	Mu-P	++	+	−	−	−	−	−
		P	−	−	−	−	−	−	−
		P-Y	+	+	−	−	−	−	−
		B	+	−	−	−	−	−	−
		Bacilli	−	−	−	−	−	−	−
Anti-white serum		Mu-Y	−	−	−	−	−	−	−
		Y	−	−	−	−	−	−	−
		W	+	++	++	+	+	+	−
		T	−	−	−	−	−	−	−
	Cocci	Mu-P	−	−	−	−	−	−	−
		P	−	−	−	−	−	−	−
		P-Y	−	−	−	−	−	−	−
		B	+	−	−	−	−	−	−
		Bacilli	−	−	−	−	−	−	−
Anti-translucent serum		Mu-Y	−	−	−	−	−	−	−
		Y	+	+	−	−	−	−	−
		W	+	+	+	±	−	−	−
		T	−	−	±	+	+	±	−
	Cocci	Mu-P	−	−	−	−	−	−	−
		P	−	−	−	−	−	−	−
		P-Y	−	−	−	−	−	−	−
		B	+	−	−	−	−	−	−
		Bacilli	+	−	−	−	−	−	−

TABLE 1—*Continued*

			DILUTION OF SERUM						
			1:10	1:20	1:40	1:80	1:160	1:320	1:640
Anti-mucoid-pink serum		Mu-Y	++++	+++	++	+	−	−	−
		Y	++	+	+	−	−	−	−
		W	+	+	+	−	−	−	−
		T	−	−	−	−	−	−	−
	Cocci	Mu-P	++++	++++	+++	+	−	−	−
		P	−	−	−	−	−	−	−
		P-Y	−	−	−	−	−	−	−
		B	++	++	+	+	+	+	−
		Bacilli	−	−	−	−	−	−	−
Anti-pink serum		Mu-Y	−	−	−	−	−	−	−
		Y	−	−	−	−	−	−	−
		W	−	−	−	−	−	−	−
		T	+	−	−	−	−	−	−
	Cocci	Mu-P	−	−	−	−	−	−	−
		P	++	++	++	+++	+++	++	+
		P-Y	+++	++	+	±	−	−	−
		B	−	−	−	−	−	−	−
		Bacilli	−	−	−	−	−	−	−
Anti-P-Y serum		Mu-Y	−	−	−	−	−	−	−
		Y	++	++	+	+	−	−	−
		W	−	−	−	−	−	−	−
		T	−	−	−	−	−	−	−
	Cocci	Mu-P	−	−	−	−	−	−	−
		P	+++	++	++	+	+	−	−
		P-Y	+++	+++	++	+	+	+	+
		B	+	+	+	−	−	−	−
		Bacilli	−	−	−	−	−	−	−
Anti-brown		Mu-Y	−	−	−	−	−	−	−
		Y	−	−	−	−	−	−	−
		W	+	+	−	−	−	−	−
		T	+	+	−	−	−	−	−
	Cocci	Mu-P	−	−	−	−	−	−	−
		P	+	+	−	−	−	−	−
		P-Y	+	−	−	−	−	−	−
		B	++	+	+	+	+	+	+
		Bacilli	−	−	−	−	−	−	−

TABLE 1—*Concluded*

			DILUTION OF SERUM						
			1:10	1:20	1:40	1:80	1:160	1:320	1:640
Anti-bacillus serum		Mu-Y	−	−	−	−	−	−	−
		Y	−	−	−	−	−	−	−
		W	−	−	−	−	−	−	−
		T	−	−	−	−	−	−	−
	Cocci	Mu-P	−	−	−	−	−	−	−
		P	−	−	−	−	−	−	−
		P-Y	−	−	−	−	−	−	−
		B	−	−	−	−	−	−	−
		Bacilli	++	++	++	++	+	+	−

++++, firm disc; +++, disc easily broken up; ++, coarse agglutination; +, fine agglutination; −, no agglutination.

antiserum and 0.2 cc. physiological saline solution. The tubes were incubated at 37°C. for 2 hours, placed in the ice-box over night and read.

RESULTS

A satisfactory agglutinating serum was prepared against each of the dissociant forms. The anti-white and anti-bacillus sera were strictly specific, agglutinating their respective forms only. The other anti-sera agglutinated their respective forms in high dilution but other forms as well, though at lower dilution. The results are shown in table 1. The anti-mucoid-yellow serum agglutinated mucoid-yellow organisms in a dilution of 1:160, mucoid-white cocci at 1:160 and the mucoid-pink organisms at 1:40 but failed to agglutinate any other variant form. Anti-yellow serum agglutinated yellow organisms in dilutions often as high as 1:640. Unexplained irregularities of agglutination were noted in experiments with yellow organisms and anti-yellow serum. At times no agglutination occurred and again, under apparently similar conditions, agglutination occurred at dilution of 1:1280. Anti-white and anti-translucent sera usually agglutinated their homologous forms only, in dilutions of 1:320 and 1:320 respectively. There was occasional cross agglutination in low dilutions of 1:10 or 1:20 with heterologous variants in the anti-yellow and translucent sera.

Anti-mucoid-pink serum caused clumping of mucoid-pink

organisms at 1:80, mucoid-yellow organisms from which the variant was derived at 1:80 though less compactly, mucoid-white cocci at 1:320, yellow and white organisms at 1:40. Mucoid-white organisms were strongly agglutinated in anti-mucoid-yellow, anti-mucoid-pink and anti-yellow sera. Of especial interest were the reactions of the anti-pink-yellow serum and pink-yellow organism. It will be recalled that this form was derived from a yellowish-buff colored sector which developed in a point pink colony, and bred true on subculture. Anti-pink-yellow serum agglutinated pink-yellow organisms in high dilution, 1:640, pink organisms at 1:160 and yellow organisms at 1:80, showing thereby an immunologic relationship between these 3 forms. Pink-yellow organisms were correspondingly agglutinated by anti-yellow serum in low dilution and in anti-pink serum at 1:80. The anti-pink serum agglutinated its homologous form at times as high as 1:2560, the pink-yellow variant at 1:80 and none other, even at the lowest dilutions. The pink organisms were highly specific and were seldom agglutinated by other antisera, except that prepared with yellow-pink forms. The anti-brown serum on the other hand was most unspecific. Brown organisms were agglutinated at high dilution, 1:2560, but other forms were frequently clumped at low dilutions. The brown organisms frequently tended to agglutinate spontaneously in all sera. Anti-bacillus serum was strictly specific, agglutinating the bacilli in dilution of 1:1280. The bacilli were not agglutinated by any other sera. Prozone phenomena were frequently noted, especially with the anti-pink serum.

Immunologic relationship between strains from other sources. In preliminary experiments, yellow or white variant forms of 16 other strains were tested with the anti-yellow and anti-white sera prepared from the strain under study. Of the six strains obtained from culture collections only one, the white variant of the strain "*ruber*," agglutinated specifically in anti-white serum. Among 10 strains isolated from patients locally the yellow variants of two, "Arens" and "Sache," were agglutinated by anti-yellow serum. Three strains therefore appeared to be similar immunologically to the strain under investigation.

In subsequent experiments the yellow, white and pink variants

of the strain "Arens" and the yellow, white, pink and brown variants of strain "*ruber*" were tested in the corresponding stock anti-sera. Surprisingly, each variant form agglutinated specifically in the anti-sera prepared from the corresponding variant of the stock strain. None of the variant forms of heterologous strains which were tested were agglutinated. It appeared therefore that among the three immunologically similar strains there was evidence of parallel immunologic specificity among the dissociants; that is, all of the corresponding variant forms of each of the three strains were similar immunologically. The results of the agglutination tests are shown in table 2.

Precipitin tests. Specific precipitation occurred when the supernatant fluid from cultures of yellow, white, pink and brown dissociants of the stock culture were added to the corresponding anti-sera of the stock strain, as shown in table 3. No precipitation occurred when the substrate from the cultures of one variant was added to the anti-serum prepared from a different variant form. There was evidence therefore that each of the 4 variant forms tested elaborated a specific soluble substance.

Phenomena similar to those observed during agglutination experiments occurred when the supernatant fluid of old broth cultures of the variant forms of the type specific "*ruber*" and "Arens" strains was added to the corresponding stock anti-sera as shown in table 3. Minor irregularities were occasionally noted during the experiments. Occasionally no precipitate occurred when the white form of "Arens" strain was tested. Occasionally precipitation occurred in anti-yellow serum when the substrate from the yellow variant of a heterologous type strain (Perkins) was used. Soluble substances from other variants of the Perkins strain failed to cause precipitation when added to any of the sera.

The pigments. Studies made of the pigments by Dr. Carl Eklund will be reported in detail in a subsequent paper. The pigments belong to the carotenoid group. Briefly it may be stated that the pigment of mucoid-yellow and yellow variants was tentatively identified as β xanthophyll or sarcinin, that of the mucoid-pink as lycopene and that of the pink form as rho-

TABLE 2

Agglutination reactions of strains from other sources as compared with the reactions of homologous cocci in immune serum prepared with variants of the strain of M. tetragenus under study

		DILUTION OF SERUM									
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Control, no serum
Anti-serum Homologous cocci	Y	−	+	+	+	+	+	−	−	−	−
	W	+	+	+	+	+	−	−	−	−	−
	P	+	+	+	+	+	+	+	+	−	−
	B	+	+	+	+	+	+	+	+	−	−
Anti-serum "Arens" cocci (homologous type)	Y	+	+	+	+	+	−	−	−	−	−
	W	+	+	+	−	−	−	−	−	−	−
	P	+	+	+	+	+	+	+	+	+	−
Anti-serum "Ruber" cocci (homologous type)	Y	+	+	+	+	−	−	−	−	−	−
	W	+	+	−	−	−	−	−	−	−	−
	P	+	+	+	+	+	+	+	+	+	−
	B	+	+	+	+	+	+	+	+	−	−
Anti-serum "Perkins" cocci (heterologous type)	Y	−	−	−	−	−	−	−	−	−	−
	W	−	−	−	−	−	−	−	−	−	−
	P	−	−	−	−	−	−	−	−	−	−

doxanthin. The pink-yellow variant appeared to elaborate 2 pigments, the absorption bands of the pink one corresponded with γ carotene or rubixanthin, while the yellow pigment appeared to be β xanthophyll or sarcinin like that of the yellow forms. One pigment of the pink-yellow form could be made to predominate over the other by cultivation at different temperature. The brown variant also contained at least 2 pigments; the pink one appeared to be identical with the described pigment of the pink-yellow form, the other is at present still unidentified.

TABLE 3

Precipitin tests of soluble substances of homologous type and heterologous type cocci

			DILUTION OF CULTURE BROTH			
			1:2	1:4	1:8	1:16
Anti-serum	Y, W, T, P, B — Homologous cocci	Y	+++	+++	++	−
		W	++++	++	+	+
		T	+	−	−	−
		P	++++	+++	++	−
		B	++++	++	+	−
Anti-serum	Y, W, P — "Arens" cocci (homologous type)	Y	+	+	+	−
		W	−	−	−	−
		P	+++	++	++	
Anti-serum	Y, W, P, B — "Perkins" cocci (heterologous type)	Y	+	−	−	−
		W	−	−	−	−
		P	−	−	−	−
		B	−	−	−	−

++++, disc formation; +++, heavy precipitate; ++, coarse precipitate; +, faint precipitate; −, no precipitate.

DISCUSSION OF PAPERS II AND III

From the limited observations at hand in earlier work on microbic dissociation, it was believed that the variant forms of a number of bacteria were limited to two or three kinds and could often be recognized by examining the character of the surface of the colonies, by the nature of the growth in broth or by testing differences in immunologic reactions or virulence. The variant forms were in some cases designated as "D" or "G" or as "S"

and "R" to denote the smooth or rough nature of the surface of the colony (Hadley, 1927). Further observation soon showed that a third and fourth form of colony variant designated as "M" (mucoid) and "G" were frequently encountered. Recently, however, in studies, especially on the pneumococcus (Eaton, 1934) and on the staphylococcus, (Hoffstadt and Youmans, 1932) numerous other variants were shown to exist bearing out many of Mellon's (1925, 1926, 1927) observations and also Hadley's (1927) statement that ". . . the free living microörganism is potentially a kaleidoscopic thing. . . ." The newly recognized variant forms were regarded either as intermediary steps between the older known variants or as separate variant forms. In the light of these recently established facts the confusion in nomenclature which arose 40 years ago and the attempts to create new species to accommodate the variants is easily understandable.

According to observations recorded here it appears that *M. tetragenus* is indeed "kaleidoscopic" especially in regard to the different brilliant pigments of the variant forms. I have isolated seven relatively stable variants—yellow, white, translucent, pink, pink-yellow, brown and a bacillary form which probably can be included as a variant form; four relatively unstable variants, small-white, mucoid-yellow, mucoid-white, and mucoid-pink, and from time to time indefinite transitional stages between the variant forms. Somewhat similar variants have been observed among staphylococci which are closely related to *M. tetragenus* (Pinner and Voldrich, 1932).

The appearance of a bacillary form was at first attributed to contamination, but its persistent appearance among single-colony cultures of the pink form alone, strongly supported its inclusion among the variant forms. Unfortunately, reversion to the coccus form or evidence of immunologic relation with the variants which would verify its recognition as such, was not observed during these experiments. Similar bacillary forms of tetrad cocci have been reported by Matzuchita (1900), Mellon (1925, 1926, 1927), and Schmidt-Kehl (1930). The latter investigator noted repeated reversion of single-cell cultures, in turn, from the coccus to the bacillus to the coccus form. The recent reports of

the occurrence of bacillary forms among pneumococci (Reimann, 1925), streptococci (Callow, 1933), and gonococci (Raven, 1934) indicate that these forms can not be dismissed lightly as contaminations due to faulty technic.

Immune sera prepared with the variant forms were usually specific for the variant used as antigen. Most of the variants were linked to one another, directly or indirectly, by specific immune similarities. The bacillus, whose inclusion as a variant may be doubtful, exhibited no immune relationship. Cross agglutination was noted between the mucoid-yellow, mucoid-white and the mucoid-pink forms and between the pink-yellow and pink and yellow forms. In the case of the mucoid-yellow and mucoid-pink the pigments of each were different. One of the pigments of the pink-yellow appeared to be identical with the pigment of the yellow form; its pink pigment was different from that of the original pink variant, but was like one of the pigments of the brown form, with which there was no immunologic relationship.

Of interest, was the evidence of type specificity among a limited number of strains obtained from widely separated sources. The antibodies evoked with the stock strain under study agglutinated specifically two of ten strains isolated locally and one strain obtained from Vienna, suggesting that type specificity akin to that of the pneumococcus, prevails in the *tetragenus* group. Of greater interest was the observation that antisera prepared with the several variant forms of the stock strain agglutinated specifically the corresponding variant forms of those strains whose yellow or white form was specifically agglutinated by the corresponding stock antiserum. This indicates, at least among the strains studied, that a specific immune parallelism exists, including even the numerous variant forms. Similar observations were made with the corresponding specific soluble substances.

It would be desirable to relate, in some way, the variant forms encountered with the trend observed in other bacteria. For example, the "M" form of other bacteria is probably represented by the mucoid-yellow, the "S" form by the yellow, the "R" form by the white and the "G" by either the small white or the translucent variety. The terminology, however, fails to account for

the mucoid-white, mucoid-pink, pink-yellow, pink, brown or bacillary variants which appear to be separate variant forms rather than intermediate stages. Another difficulty arises in regard to the existence of three "mucoid" variants, mucoid-white, mucoid-yellow and mucoid-pink in the same dissociative pattern. The other variants, unlike those of the pneumococcus, for example, do not appear to be "degraded" cells but in some instances actually appear to be more resistant and vigorous in some respects than what might be regarded as the original white form, as will be shown in a succeeding paper. Unlike certain other bacteria, growth in broth containing homologous immune serum and in lithium chloride broth was not as effective in enforcing dissociation as other means. Each of the variant forms tested evoked specific homologous agglutinins in fairly high titre, in contrast with the pneumococcus whose M form is strictly type specific, but whose S and R forms are merely species specific.

The question of the existence of a life-cycle was considered. This matter has been thoroughly discussed by Mellon, Hadley and more recently by Winslow (1935). In studies of *M. tetragenus* it does not seem necessary to conceive of an obligate life-cycle in the sense of that observed among malaria plasmodia, butterflies or among higher plants in which a complete transformation through the pupa or seed stage, for example, is obligatory to the continuance of existence. It appears, under laboratory conditions which are admittedly highly artificial, that, given favorable circumstances, any of the seven "stable" forms may reproduce their own kind through an infinite number of transfers. In the earlier period of experimentation with *M. tetragenus* it appeared that a kind of "cycle" existed among the yellow, white and small white and translucent stage as observed by ageing cultures on agar plates. On further study, under a variety of conditions *in vitro* and *in vivo*, the appearance of numerous other variant forms failed to support the theory of a life-cycle of this organism in the strict sense of the term except that under experimental manipulation the following sequence was occasionally noted, which might be accepted as representing a life-cycle,— Y → W → small-W → T → P → W → Y. Mellon regards

the phenomena in the following light. ". . . Each characteristic pleomorphic phase of a microörganism is capable of being stabilized as such; the culture thereafter multiplying what was formerly its pleomorphic form. It is as if the protoplasm were cast into a new mold and in order for the new form to 'set' it must pass through a transforming vehicle. The latter is represented by one or another of the reproductive forms of the bacteria and it is from these that we expect the new morphologic type to germinate."[2]

The sequential appearance of the various variant forms as they were unfolded from the "white" form originally obtained from the patient, is illustrated in figure 1. The white form easily changed into the yellow, but with more difficulty into the small-white which, in turn, appeared to give rise to the translucent form. Under relatively drastic treatment or unfavorable circumstances, both *in vitro* and *in vivo*, the translucent gave rise to the pink, to the brown, and in turn to the bacillary form. The three mucoid forms were encountered only after many months of observation. They appeared in two ways, first by ageing their respective yellow, white and pink forms in broth culture, and also by sector formation, that is, mucoid-pink colonies appeared to arise from the mucoid-yellow ones on agar plates and the mucoid-pink in turn gave rise to the mucoid-white. The three mucoid forms were closely related immunologically. The pink-yellow variant appeared as a sector in a point pink colony. The yellow, white, pink and brown when transferred to fresh agar at intervals of several days or weeks, depending upon the temperature, reproduced their own kind for more than 70 transfers indicating a relative stability when the environment is satisfactory, as schematically illustrated by the vertical lines in figure 1. The "*ruber*" strain obtained from Vienna has been propagated, for example, in a culture collection in its pink form for 42 years. On the other hand, various methods of treatment provoked the appearance of other dissociate forms illustrated by the short lines branching off the main ones in figure 1. No regularity of

[2] Personal communication to the author.

transformation was noted except that the principle trend appeared to favor reversion toward the yellow and white forms.

The less stable forms of mucoid-yellow and mucoid-pink tended to revert to the original yellow or pink forms. The small-white colonies produced both large and small-white, yellow or translucent, and like the translucent form were unstable and gave rise to the greatest variety of forms. The mucoid-white frequently reverted to the mucoid-pink or white forms.

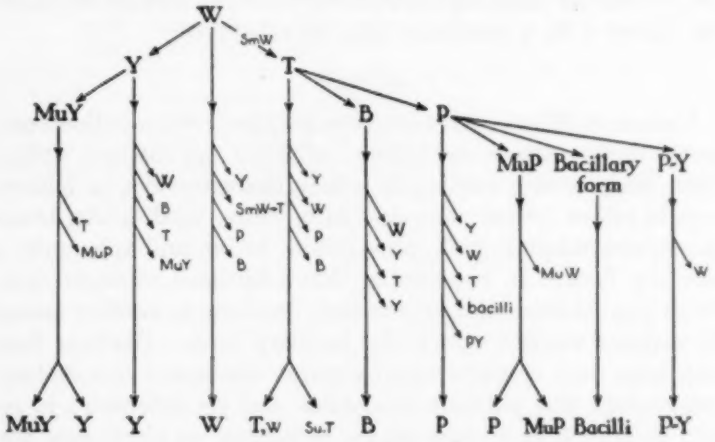

FIG. 1. SCHEMATIC REPRESENTATION OF THE ORDER OF APPEARANCE OF THE VARIANT FORMS OF THE STRAIN OF M. TETRAGENUS UNDER STUDY

The white form as derived from the blood of a patient gave rise to the various forms shown. The general trend of growth of each form under favorable conditions is represented by descending vertical lines. Short branches indicate the development of variants.

The translucent form, after a number of transfers on agar, gradually became more and more opaque, less hemolytic and finally changed into the white or yellow form. Numerous autolytic or suicide colonies frequently appeared and occasionally composed the entire flora of the plate. As these colonies appeared to melt away, numbers of minute daughter colonies often appeared and remained viable after the original colony had vanished. The significance of these autolytic variants is unknown. A study of a somewhat similar phenomenon among pneumococci has recently been reported (Eaton, 1934).

None of the variant forms studied were pathogenic for mice or rabbits. Capsules did not, therefore, endow the mucoid-yellow, mucoid-white or mucoid-pink forms with virulence. On the other hand virulence of the two strains of *M. tetragenus* obtained from Munich was associated with capsules and the organisms grew in mucoid-white colonies. The situation appears to be somewhat analogous to that described by Julianelle and Wieghard (1935) in the closely related staphylococci, among which virulence is not necessarily associated with the presence of soluble substance but rather with a particular type of substance.

RÉSUMÉ

A strain of *Micrococcus tetragenus* obtained from a patient, and grown under various conditions, exhibited ten distinct variant forms designated according to colony characteristics as follows: mucoid-yellow, yellow, mucoid-white, white, small-white, translucent, mucoid-pink, pink, pink-yellow, brown and apparently a bacillary form. It is probable that additional variants exist. There was evidence of reversion from one form to another among all variants studied except the bacillary form. Bacteria from each form were characterized by rather distinctive morphology, immunology and pigment production and by differences in regard to growth in various sugars, in gelatin, on blood agar and in ability to grow over a wide range of temperature and hydrogen ion concentration changes. None of the variant forms were virulent for white mice, guinea pigs or rabbits. The pigments elaborated by the colored forms belonged to the carotenoid group, and are still under investigation. Each of the variant forms evoked specific agglutinating and precipitating sera which were found to agglutinate specifically the corresponding dissociant forms of three homologous type-specific strains of *Micrococcus tetragenus* obtained from other sources.

The pattern of dissociation observed has been compared with that of other bacterial species and the question of life-cycles briefly discussed.

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STUDIES ON HEMOLYTIC STREPTOCOCCI

I. METHODS OF CLASSIFICATION

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INTRODUCTION

That a satisfactory method for the classification of the hemolytic streptococci has not yet come into practical use is illustrated in the last edition (1934) of Bergey's Manual of Determinative Bacteriology, by the description of six species, *pyogenes*, *scarlatinae*, *puerperalis*, *erysipelatis*, *epidemicus* and *cuniculi*, with no distinctive characters other than that they are said to cause different diseases, and that, for some, specific serologic reactions are claimed.

Ability to produce a specific disease is a character which may be utilized in bacterial classification, in so far as it may contribute to the differentiation of species. The fact is now well established, however, by the works of many investigators, and especially by the agglutinin absorption studies of Williams and her collaborators, and also those of Griffith, that a given serological type of streptococcus may cause more than one kind of disease.

There is also abundant epidemiological evidence that a given strain of streptococcus can cause more than one kind of disease, in the many reports to be found in the literature of more than one streptococcus disease occurring in the same epidemic. For example, among 50 septic sore throat epidemics reported by Williams, cases of scarlet fever occurred in 18, and cases of erysipelas occurred in 11. Hence it is obvious that an attempted differentiation of streptococci into species on the basis of disease source ends in confusion.

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Thompson and Megrail recently reported a study of 120 strains of streptococci, in which the correlation between type of hemolysis on blood agar and certain biophysical, biochemical and pathogenic properties of the strains was investigated. They failed to find a test which would differentiate the hemolytic streptococci into smaller groups of significant meaning. According to Zinsser and Bayne-Jones (1934) the material for a natural classification of streptococci is not yet available.

Much valuable information has been obtained, however, by individuals, or by small groups working with one or more tests, or working with strains from limited sources. To bring together in a large collection representative strains from the collections on which studies have been made, and to coördinate the work of the many investigators into a rational classification was the object of the present study. The tests which other investigators have found useful for classification were considered, and the strains were also studied from a new point of view dependent on their sensitivity to several races of streptococcus bacteriophage.

SOURCE OF CULTURES

Strains from the following sources have been studied:

	Number of strains
Animal sources:	
Various diseases	139
Suspected milk associated with epidemics	14
Human sources:	
Scarlet fever	119
Puerperal fever	62
Erysipelas	38
Epidemic sore throat	32
Rheumatism	16
Acute suppurative diseases[1]	52
Other miscellaneous diseases	56
Normal throats	30
Intestinal and other miscellaneous sources	15
Total	573

The streptococci were isolated in distant parts of the world. The writer is greatly indebted to the many investigators who re-

[1] The acute suppurative diseases include empyema, osteomyelitis, peritonitis, mastoiditis, meningitis, sinusitis, otitis media, cystitis, adenitis and abscesses.

sponded to requests for representative strains of their collections. Without the coöperation which was so generously given, the present studies could not have been made.

In determining the relationship between bacterial species and specific diseases, it is of great importance that strains from distant localities should be compared. The data collected in the present study show a number of instances in which an unusual type of streptococcus was disseminated throughout a limited area; and there are in the literature erroneous conclusions as to the disease specificity of certain types of streptococci which would have been avoided if strains collected from wider areas had been studied.

In anticipation of the forthcoming series of papers it may be stated that several species with peculiar pathogenic properties are recognizable. One is capable of causing erysipelas and no other disease, in so far as our data show; another causes scarlet fever, and apparently no other disease. The understanding of their disease specificity has been confused, however, by the fact that there are other species which may cause erysipelas, or scarlet fever or various other kinds of infections. The results of this study reconcile what appeared to be the opposing opinions of those on the one hand, who contended that the streptococci of scarlet fever and of erysipelas are disease specific; and of those, on the other hand, who contended that the two diseases are caused by the same organism.

CHOICE OF DIFFERENTIAL CRITERIA

In order to be practical, the characters utilized for routine identification of streptococci must be correlated with pathogenic properties; and in order to be most useful, they should be as simple as possible.

Some of the characters of streptococci which are of greatest interest when their pathogenic properties are considered are of little value for routine classification purposes, because differences are quantitative, with every gradation between the extremes. Thus, according to our present knowledge, one qualitative type of leucocidin is produced in varying quantities by more than one

species of streptococcus Likewise, according to Van Deventer, there is only one immunological type of streptococcus when judged by the fibrinolytic property. Todd reported that the hemolysin produced by animal strains of hemolytic streptococci differed serologically from that of human strains, but Loewenthal and Pradham found only quantitative differences.

Virulence for mice is another character in which differences are quantitative, varying from the high degree generally possessed by certain groups which cause spontaneous disease in animals, to the low degree possessed characteristically by certain groups of human strains, with gradations between the groups and within each group.

On the other hand there appear to be qualitative differences in the erythrogenic toxin produced by different hemolytic streptococci capable of causing human disease (Birkhaug; Dick and Dick; Pilot and Dreyer; Trask and Blake; Hooker and Follensby; Wadsworth and Coffey). As yet, however, the difficulty of obtaining a supply of suitable sensitive experimental animals retards progress in the comparative study of toxins. Through the courtesy of those investigators who have studied the different types of streptococcus toxin the proffered scheme of classification has been correlated with toxin production in a number of strains.

THE IMPORTANCE OF SEROLOGICAL GROUPING IN A SCHEME OF CLASSIFICATION

To be accepted as logical, the classification of hemolytic streptococci cannot be discordant with the serological groupings which have been worked out. The generous coöperation of those investigators who have made comprehensive studies of agglutinin absorption and precipitin reactions has made it possible to correlate serological grouping with the characters determined in the present study, without a duplication of efforts. Further comparative studies will be necessary, however, to complete the description of characters of certain groups. Coördination of the present study with the groupings according to agglutinin absorption reactions was made possible by the addition to our collection

of the various type strains described by Williams and her collaborators; by Griffith; and by Mueller and Klise.

Coördination of the present study with the groupings according to Lancefield's precipitin test was made possible by the addition to our collection of many strains which had been previously studied by Lancefield (1933); by Edwards (1934); and by Plummer. With very generous courtesy, Dr. Lancefield has typed a few strains of our collection which represent phagological groups not included in her previous studies.

The serological studies of the investigators mentioned have yielded valuable information about the grouping of streptococci. Lancefield has differentiated the hemolytic streptococci into seven groups according to precipitin reactions, and she is investigating the further subdivision of the groups. The studies of Smith; of Williams and her collaborators; of Mueller and Klise; and of Griffith have agreed in showing that the agglutinin absorption reactions divide into many types the streptococci capable of causing a given disease. For example, the scarlet fever strains studied by Griffith fall into 20 serological types.

It would be illogical to regard as distinct species those serological types which are indistinguishable except for agglutinative differences. However, on account of the division of streptococci into many types by agglutinin absorption reactions, that test is most useful for tracing epidemics to their sources when time and circumstances permit an exhaustive study. On the other hand, the exigencies of an epidemic demand a more simple and speedy test which will classify strains into groups harmonious with the serological groups.

THE RELIABILITY OF FERMENTATION AND OTHER REACTIONS

The reliability of fermentation reactions for the differentiation of hemolytic streptococci has been considered in recent reviews of the literature. The Thomsons conclude that it would be dangerous to classify streptococci primarily on the basis of sugar reactions, because various investigators use different methods which give different results. There is another aspect of the reliability of fermentation tests for the classification of streptococci—con-

cerned with the question whether differences in the fermentation of a given carbohydrate should be regarded as belonging to the category of species, variety or race differentiation. This consideration is recognized by Okell, who concludes that fermentation tests are useful in identifying particular individual strains, but that they fail to supply a method of classification of pathological importance. Williams thinks that fermentation reactions should be used as a basis for the study of new strains until some other method is shown to be better. The studies of Ogura, of Edwards (1933) and of Minett showed that human strains of hemolytic streptococci are characterized by the fermentation of trehalose but not sorbitol, whereas animal strains generally ferment sorbitol but not trehalose.

In our experience, after studying the correlation of fermentation reactions with other reactions and with the sources of the strains, we have arrived at the conclusion that the ability to ferment certain substances is of definite value for the classification of hemolytic streptococci. Differences revealed by reactions in the presence of those substances are so well correlated with other distinguishing characters that they may be regarded as specific.

Final pH in glucose broth was proposed by Ayers, Johnson and Davis as a character for the differentiation of streptococci. Their observation that non-pathogenic bovine strains produce a higher final acidity than pathogenic strains was confirmed and amplified by Avery and Cullen, and by many subsequent investigators.

Ayers and Rupp devised another test which differentiates "*Streptococcus pyogenes*" from *Streptococcus mastitidis*. They found that the latter will hydrolyze hippuric acid with the formation of benzoic acid and glycocoll. The benzoic acid is readily detected in the medium by the addition of ferric chloride. This observation has been confirmed by many investigators.

Capsule formation was not used as a differential test in this study because many investigators (Rosenow; Ward and Lyons; and others) have shown that a given strain may develop capsules in one environment but not in another.

Colonial appearance was not considered in this study because

it has been established by the observations of Cowan and many subsequent investigators that a given strain may show variations in type of colony.

The cultivation of streptococci on benzidine blood agar is advocated by Tunnicliff for the identification of scarlet fever strains. She states, however, that some of the dissociants of scarlatinal streptococci behave like erysipelas and sore throat strains in producing a black color in this medium. In our experiments certain erysipelas and sore throat strains produced a marked blackening of the medium, in contrast to certain other strains which did not produce this effect. The scarlet fever strains, however, were not consistent in their behavior on this medium. Our results with hemolytic streptococci from a great variety of disease sources, representing every species in our collection, led to the conclusion that the test lacks the precision necessary for a differential test. Possibly the fact that old stock cultures were used in our studies, whereas Tunnicliff apparently studied freshly isolated strains, may account for the discrepancy in our results.

SENSITIVITY TO BACTERIOPHAGE AS A DIFFERENTIAL TEST

Bail was the first to suggest that sensitivity to bacteriophage might serve as a means for the differentiation of closely related bacteria. It has never been so used in any extensive classification study, presumably because those races of phage which have been subject to the most extended studies—the staphylococcus, *Salmonella*, dysentery and cholera phages—are too broad in their lytic capacities to permit differentiation of bacterial species.

Recently, however, Levine and Frisch studied the possibility of classifying *Salmonella* according to the ability of the heat-killed organisms to absorb bacteriophage. They were unable to differentiate the organisms of the *typhosus-enteritidis* group and those of the *aertrycke-paratyphosus* B groups from each other, although the results of a previous study by these authors, and also the investigations of Gough and Burnet, who worked with the dysentery bacilli, indicate that the specific somatic antigen is a complex labile polysaccharide which is responsible for both the serologic character and the phage susceptibility of the bacteria.

Using a different phagological technique from that employed by Levine and Frisch, the writer has found that sensitivity to several phages offers a key to species differentiation of streptococci, with phagological groupings in agreement with serological groupings. In a previous publication (Evans, 1935), it was reported that sensitivity to several races of phage in the nascent stage, that is, in the presence of a sensitive strain, is a distinctive character of hemolytic streptococci. Previous to that report, Kendrick and Hollon had noted briefly that there is a definite parallelism between the serologic and bacteriophagic reactions of the fecal streptococci; and Lancefield (1932) had reported that 47 out of 56 strains of streptococci found to be sensitive to the Clark phage (B filtrate) were of animal origin and belonged to her serological group C.

The technique described further on for the classification of hemolytic streptococci according to phage sensitivity is reasonably simple. The phages retain their potency indefinitely when kept in a refrigerator. Hence, the stock supply is always ready for instant use, and the reactions may be determined overnight. Four phage preparations are sufficient for the classification of all hemolytic streptococci.

On the whole, the groupings made with the aid of sensitivity to phage as a differential character agree with species which have already been made on other bases. A few new groups have emerged in this study, however, which appear to be so well defined that they should be regarded as species not hitherto recognized. Further, the study has shown that certain names which have been applied to alleged groups with no other distinctive character than the ability to produce a certain disease, do not apply to distinct species and must be discarded or regarded as synonyms in a logical scheme of classification.

CHARACTERS INCLUDED IN THE PRESENT STUDY

The present study includes the determination of the following characters for all strains of the collection: Sensitivity to streptococcus phages A, B, C and D; final pH in glucose broth; fermentation of lactose, salicin, mannitol, trehalose and sorbitol; ability

to grow on blood agar containing 10 per cent bile; ability to grow on blood containing 40 per cent bile; and hydrolysis of sodium hippurate.

The tests mentioned above served to classify the strains into groups. Further studies, including the fibrinolytic reaction and virulence for mice were made on representative strains of the various groups. The ability to produce agglutinins in rabbits was determined for some of the strains belonging to the species *Streptococcus equi* and related groups; and the ability to produce erysipelas in rabbits was determined for representative strains derived from human erysipelatous lesions.

DIFFERENTIAL TESTS

The tests which were applied to all strains of the collection did not prove to have equal differential value. The well-defined groups are differentiated by the following characters: Sensitivity to phages A, B, C and D in the nascent state; sensitivity to phage B filtrate; and fermentation reactions in lactose, salicin, mannitol, trehalose and sorbitol broths. These will be called the differential tests; and for convenience of discussion, the present scheme of classification will be called the phagological classification, though the 5 mentioned fermentation reactions are also important in the scheme.

A highly acid final reaction in glucose broth merely corroborates distinctions clearly made by the differential tests. The two species which hydrolyze sodium hippurate, are clearly distinguished by the primary differential tests. Growth on 10-per cent bile blood agar was found to have no differential value. Growth on 40-per cent bile blood agar was found to be so well correlated with the reactions of the differential tests that the bacteriostatic action of bile merely adds confirmatory evidence.

Phage A was found to be so broad in its activity that it is useful only in the case of three groups to corroborate differences otherwise shown by the differential tests. It was pointed out in an earlier paper (1935) that by the use of 2 strains of phage C, certain groups intermediary between *S. pyogenes* and *S. epidemicus* may be distinguished. The use of both strains of phage C is recommended for the differentiation of those species.

EXPERIMENTAL PROCEDURES

The strains of streptococci included in this study are aerobic, and produce a zone of clear hemolysis when smeared on blood agar plates, (infusion agar containing 5 per cent of rabbit blood) and incubated 48 hours before examination.

Sensitivity to phage. The four races of streptococcus phage used in these studies, A, B, C and D were described in an earlier paper (Evans 1934) in which the histories of the strains of streptococci used for substrata were also given. Lytic filtrates are designated by a letter for the race of phage and a number which designates the strain of streptococcus which served as a substratum, as, for example, A/751.

It was stated in the earlier publication (1934) that the range of activity of a given race of phage is influenced by the strain of streptococcus which serves as the substratum for its multiplication. Hence it was necessary to choose for the propagation of each race of phage a strain of streptococcus which would give it such range of activity as would be most useful for classification purposes. The two races of phage, B and D, which were received from other laboratories, were each propagated on the strain of streptococcus which was used as a substratum by the preceding investigators. For the two races of phage isolated in this laboratory, A and C, a considerable time was spent in making an effort to choose suitable streptococci for substrata. The effort was without success in the case of phage A. The results with strain 751 for a substratum seemed promising, however, and all strains were tested for sensitivity to nascent phage A/751. As already stated, the range of activity of this phage proved to be too broad to be useful for classification purposes.

In the case of phage C it was found that two strains of streptococci, 594 and 646 would each impart to the phage a distinct range of activity useful for the classification. It would be a comparatively easy matter now, after the classification studies have been made, to select appropriate strains for use as substrata for phage C. But the selections had to be made previous to the classification studies, when the characters of relatively few of

the strains were known. Hence the procedure was largely one of trial and error which need not be described in detail.

It was found that sensitivity to lytic filtrate B/563 was a useful character for classification purposes, as well as sensitivity to B/563 in the nascent state. Tests were therefore carried out with each strain of the collection for sensitivity to phages A/751, B/563, C/594, C/646 and D/693 in the nascent state, and for sensitivity to lytic filtrate B/563.

The stock supply of lytic filtrates was prepared as follows: Tubes containing about 10 cc. of broth, were each planted with one drop of sensitive culture and one drop of lytic filtrate of the phage to be prepared. The contents of the tubes in which clearing occurred were filtered together through a Berkefeld N filter, tubed, and stored in the refrigerator for use.

The test for sensitivity of a streptococcus strain to one of the races of phage in the nascent state can be most clearly described by an example. In testing *Streptococcus* 599 for sensitivity to phage A/751, a drop of diluted filtrate A/751 and a drop of culture 751 are added to each of 2 tubes of broth. One of these tubes serves for the phage control. A control tube of broth is also planted with a drop of culture 751 alone. Then one of the tubes containing the diluted phage and culture, and a tube of sterile broth are each planted with a drop of culture 599. All the tubes are incubated overnight and examined in the morning. If the broth cultures 751 and 599 are turbid and if the phage control tube is clear, readings are recorded. If the tube containing phage and planted with culture 599 is clear, or if it is definitely less turbid than the broth control, the reaction is recorded as positive; if the culture of 599 plus phage and the broth control culture 599 are equally turbid the reaction is recorded as negative.

The test for sensitivity to the other races of phage follows the same technique, excepting that other strains of streptococci serve as substrata.

Sometimes certain streptococcus strains give poor growth in plain broth. In that case the phagological reactions may be determined in broth containing 0.5 per cent glucose.

If there is any dissatisfaction with the reaction obtained in

the first test for sensitivity of a given strain of streptococcus to a given race of phage, the test is repeated. For example, dissatisfaction might arise if the culture containing phage was questionably less turbid than the broth control. Or, as another example, dissatisfaction might arise in comparing several strains of an epidemic if one of the strains which was otherwise like the epidemic strains disagreed in its sensitivity towards only one of the phages.

In repeating a test the procedure is exactly as described above, excepting that triplicate tests are made in different dilutions of phage (as, 10^{-1}, 10^{-2} and 10^{-3}) with phage controls for each dilution. In this study, negative results were always confirmed by triplicate tests if the identification of the strain was dependent on the reaction in question.

Fermentation reactions. The cultures were grown for 4 days in sugar-free beef infusion broth of pH 7.4 containing 1 per cent of the test substance. Tests for fermentation were made by adding 0.5 cc. of culture to 8.5 cc. of distilled water, with brom-thymol-blue as indicator, excepting for the determination of the final pH in glucose broth, for which methyl red was used. If the reaction as indicated by brom-thymol-blue was more acid than pH 6.6 it was recorded with a positive sign; if it was pH 6.6 or higher the reading was recorded.

Growth on bile blood agar. All cultures were tested for ability to grow on infusion agar containing 5 per cent rabbit blood and 10 per cent bile. The bile was prepared with dehydrated "bacto-oxgall" (Digestive Ferments Company) made up to the original density. Those cultures which grew on 10 per cent bile were tested again for ability to grow on blood agar containing 40 per cent bile. Control plates of blood agar without the addition of bile were always inoculated at the same time to insure the viability of the inoculum. Readings were made after 48 hours incubation.

Hydrolysis of sodium hippurate. The strains were grown for 4 days in infusion broth containing 1 per cent sodium hippurate. Reduction of the hippurate to benzoic acid was detected by the addition of 12 per cent ferric chloride solution containing 2.5 cc.

concentrated hydrochloric acid per liter. To 1-cc. amounts of the supernatant culture fluid 0.5 cc. of the ferric chloride solution was added and the tubes were thoroughly shaken immediately. The uninoculated sodium hippurate broth control always gave complete clearing. A precipitate which failed to disappear when the tubes were shaken was regarded as evidence of the reduction of the hippurate by the growing organisms.

A number of strains of each species were tested for ability to dissolve human fibrin, according to the technique of Tillett and Garner. Their technique was slightly modified, because when it was followed exactly, the diluted plasma did not always clot.

The tests were always carried out in duplicate, with blood from 2 normal human subjects. The plasma was prepared as follows: Twelve cubic centimeters of blood were taken from a normal person and placed in a tube containing the powder obtained by evaporating 1 cc. of a 2 per cent solution of potassium oxalate. The blood cells were separated from the plasma by centrifugation.

The streptococci to be tested for fibrinolysin were grown overnight in beef infusion broth containing 10 per cent rabbit serum. The cultures were centrifugated and the clear supernatant fluid was used for the test. In carrying out the tests, sterile conditions were maintained throughout. 0.2 cc. of plasma was added to 0.8 cc. of saline solution, 0.5 cc. of supernatant fluid from the culture was added, the tube was shaken, then 0.25 cc. of a 0.25 per cent solution of calcium chloride was added and the tube was shaken again. The tubes were incubated in a water bath at 37°C., and readings were made after 30 minutes, and after 1, 3 and 24 hours. Readings were recorded according to the following scheme:

Inhibition of clotting	++++
Complete lysis in 1 hour	+++
Complete lysis in 3 hours	++
Complete or partial lysis in 24 hours	+
No lysis	−

VIRULENCE FOR MICE

The streptococci to be tested for virulence were grown overnight in broth containing 10 per cent of rabbit's blood. White

mice weighing about 20 grams were the test animals. They were inoculated intraperitoneally with 1 cc. of the suitably diluted culture.

A preliminary test was made with the 1:100 dilution of culture. If the mouse survived 4 days, no further tests were made with that strain. If the mouse died within 4 days, the heart blood was planted on blood agar plate. If no streptococcus colonies grew on the plate, it was assumed that death was due to some extraneous cause. If a streptococcus culture was obtained, mice were inoculated with the passage culture in dilutions 10^{-3}, 10^{-5} and 10^{-7}. The results with this series determined the dilutions of the second passage culture to be used for the next series of mice. For example, if only the mouse receiving the lowest dilution died, the second passage culture was tested for virulence in dilutions 10^{-4} and 10^{-5}. Or if all 3 mice receiving the first passage culture died, the second passage culture was tested in the 10^{-3} dilution alone. Tests were not carried out with dilutions higher than 10^{-3}.

SUMMARY

The methods used for the study of 573 strains of hemolytic streptococci are described; the value of the various tests for classification purposes is discussed; and a few relatively simple tests are selected by which unknown strains may be identified with species to be described in subsequent papers of this series.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

TEMPLE UNIVERSITY SCHOOL OF MEDICINE, PHILADELPHIA, PENNSYLVANIA, JANUARY 28, 1936

THE ADVANTAGES OF VACUUM DRIED COMPLEMENT FOR USE IN THE ROUTINE WASSERMANN REACTION. *Fred Boerner and Marguerite Lukens,* Graduate Hospital, Philadelphia, Pa.

Complement dehydrated in the frozen state using the Flosdorf-Mudd apparatus was shown to retain its hemolytic activity for at least fifty-two weeks when stored in the refrigerator at 8° to 10°C. It was also shown that complement fifty-six weeks old which had lost considerable of its hemolytic activity was still as flexible as fresh complement.

The term "lyophile" has been applied to this dehydrated product. This "lyophile" complement was used routinely three times a week for over a year and one-half, during which time 12,175 sera and 675 spinal fluids were tested. The "lyophile" complement proved in all respects equivalent to fresh complement. The authors have discontinued their guinea pig colony because of the greater convenience and uniformity of this type of complement.

THE KINETICS OF LYSIS OF ESCHERICHIA COLI

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The kinetics of lysis of *Staphylococcus aureus* cultures by an antistaphylococcus bacteriophage have been studied by Krueger and Northrop (1930). These investigators reported that phage formation is logarithmic with time during the logarithmic period of the growth of *Staphylococcus aureus*; that phage formation proceeds at a much faster rate than bacterial multiplication; that extracellular and intracellular phage are distributed on a constant percentage basis; that lysis begins when a definite ratio between the concentrations of phage and bacteria is established in the cultures; that the lytic destruction of bacteria is logarithmic with time; and that the major portion of phage present after the completion of bacteriophagy is set free during the brief period of bacterial dissolution.

The studies reported in this paper were undertaken to determine whether or not similar relationships exist in cultures of *Escherichia coli* in the presence of an anticoli bacteriophage.

EXPERIMENTAL METHODS AND RESULTS

A stock phage suspension was prepared by diluting an anticoli phage (Phage 65) with Martin's broth so that it contained 1.0×10^7 phage units per milliliter, a phage unit being defined as the least quantity of phage which will always produce complete clearing of the freshly prepared phage-susceptible bacterial culture within 24 hours. This stock phage was stored at refrigerator temperature and frequent control tests showed that its activity remained constant during the course of the studies to be reported here.

The phage suspensions employed in these tests were inoculated with 20-hour Martin's broth cultures of *E. coli* (K-13), the initial bacterial concentration in the mixtures being 0.5×10^8 cells per milliliter unless otherwise stated. All mixtures were adjusted to an initial volume of 100 ml., Martin's broth (a digest broth prepared from pigs' stomachs, pH 7.6) being employed as the culture medium throughout these tests. The various cultures were incubated in a water bath at 32°C. and continuously but gently stirred by means of Ridley's (1928) mixing apparatus, the suspensions being alternately pulled into and forced out of 25-ml. pipettes supported by rubber stoppers in one neck of the 250-ml. Woulff bottles exmployed as the culture vessels.

Four-milliliter samples were removed at appropriate intervals of time (7.5 minutes or longer) and the concentrations of bacteria in the cultures were determined with the aid of a photo-electric turbidimeter recently described by Clifton, Mueller and Rogers (1935). This instrument was calibrated against different dilutions of suspensions of *E. coli*, the total bacterial count in these suspensions being determined with the aid of a Petroff-Hauser counting chamber. With a little practice a determination of the total bacterial count in a culture can be made in less than 1 minute, duplicate determinations agreeing within less than 1 per cent of each other.

One-milliliter samples of the stock phage, or of decimal dilutions thereof, were added to 99-ml. portions of Martin's broth seeded to contain 0.5×10^8 bacteria per milliliter. Growth occurred in all dilutions of the phage, the turbidity of the cultures increasing rapidly after a short lag period. The presence of phage did not noticeably alter the rate of growth from that observed in phage-free cultures. At a certain point the rate of growth decreased in the cultures containing phage, the time at which the decreased rate of growth became apparent being controlled by the initial concentration of phage. Lysis of the cells soon occurred and proceeded rapidly to completion. Typical time-growth and time-lysis relationships observed in these cultures are recorded graphically in figure 1.

Under uniform cultural conditions the time required for lysis is a function of the initial concentration of phage, this relationship furnishing a basis for the quantitative estimation of phage (Krueger, 1930). Since it is difficult to determine the exact time at which lysis is complete one chooses an arbitrary endpoint which can be accurately observed and determines the time required for lysis to reduce the concentration of bacteria to this

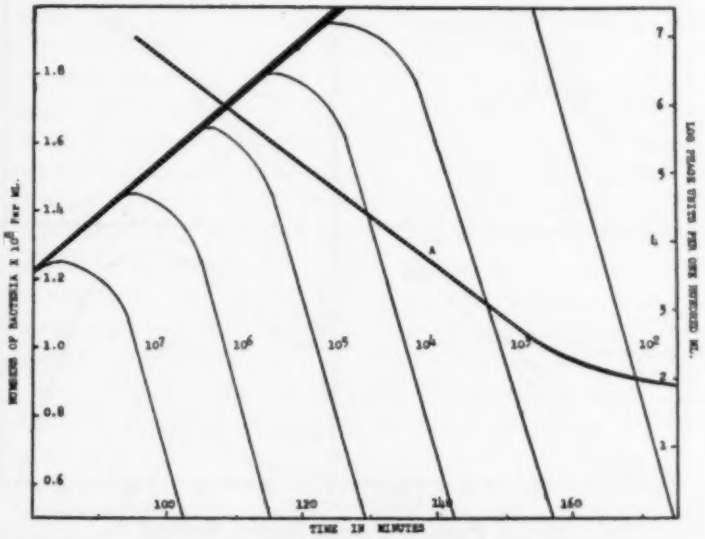

FIG. 1. TIME-GROWTH AND TIME-LYSIS RELATIONSHIPS OBSERVED IN CULTURES OF E. COLI IN THE PRESENCE OF DECIMAL DILUTIONS OF AN ANTICOLI PHAGE

The heavy line (*A*) represents the relationship between the initial phage concentration and the time required to reduce the bacterial concentration to 1.0×10^8 bacteria per milliliter under uniform conditions.

level. When the time, required to reduce the concentration of bacteria in these cultures of *E. coli* to an arbitrary endpoint of 1.0×10^8 bacteria per milliliter, is plotted against the initial concentration of the anticoli phage a straight line relationship is observed over a wide phage concentration range as is shown by the heavy line (*A*) in figure 1.

The concentration of phage in a given sample was determined

as follows. One milliliter of the phage suspension (or of an appropriate dilution) was added to 99 ml. of Martin's broth, seeded to contain 0.5 × 10^8 bacteria per milliliter. The concentration of bacteria was determined at various intervals of time, determinations being made every 7.5 minutes after lysis was initiated so that at least three points could be established for plotting residual bacterial concentrations against time during

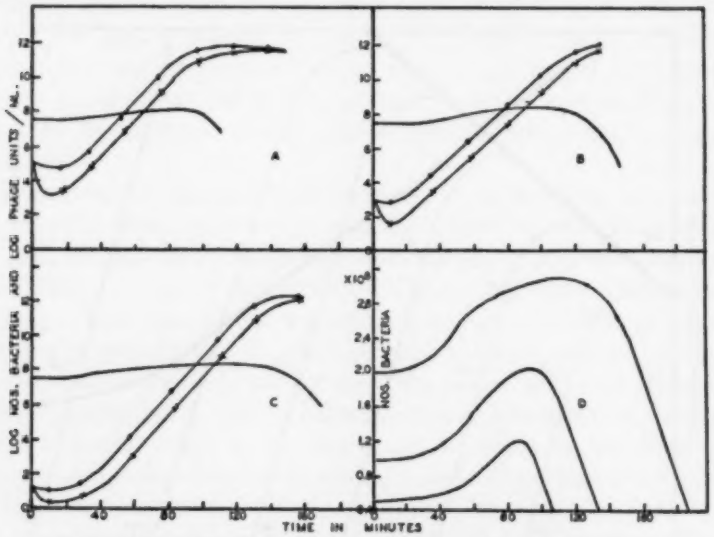

FIG. 2. PLOTS OF TYPICAL KINETIC STUDIES

Log "total" phage •———•, log "extracellular" phage x———x and log bacterial concentrations ——— against time for different initial concentrations of phage, 1 × 10^5, 1 × 10^3 and 1 × 10^1 phage units per milliliter in *A*, *B*, and *C*, respectively. *D*. Numbers of bacteria against time for different initial concentrations of bacteria (0.5, 1.0 and 2.0 × 10^8 bacteria per milliliter) and the same concentration (1 × 10^7 phage units per 100 ml.) of phage.

the period of lysis. The total time required for lysis to reach the arbitrary endpoint (here 1.0 × 10^8 bacteria per milliliter) was determined from a plot of the concentrations of bacteria against time. This time value was located on the heavy line in figure 1, the logarithm of the initial phage concentration in 100 ml. of the culture being read directly on the ordinate. This value is also equal to the logarithm of the phage concentration in the

1.0-ml. sample of phage initially added to the 99 ml. of the bacterial culture. Duplicate tests indicate that the accuracy of this method lies within a range of less than ±5 per cent.

In other experiments samples of the cultures were removed at various intervals of times for the determination of the concentrations of "total" and of "extracellular" phage in addition to the determination of the bacterial concentrations. The concentration of "total" phage in a sample was determined on the basis of the time required for lysis to reach the arbitrary endpoint after 1.0 ml. of the culture, or of an appropriate dilution, thereof, was added to 99 ml. of the standard cultures employed in these tests. "Extracellular" phage was determined in the same manner, the bacteria in the samples being first removed by filtration through L 3 Chamberland candles. Although these methods are subject to a slight "time" error and the possible removal of some phage during the filtration process, determinations with similar samples in different cultures gave results of the same order of magnitude. A control test, carried out with a known quantity of phage, was always included in these determinations. Typical results are presented in figure 2 (*A*, *B*, *C*) in which the logarithms of the concentrations per milliliter of bacteria and of "total" and of "extracellular" phage are plotted against time.

DISCUSSION

The results of this study indicate that the rate of growth of bacteria in the presence of phage does not differ appreciably from that observed in phage-free cultures, unless the initial concentration ratio of phage to bacteria is rather large (fig. 1). Normal growth is observed almost to the time at which lysis is initiated, the age of the culture when lysis commences being controlled by the initial concentration of phage and of bacteria. The rate of lytic destruction of the cells is extremely rapid as compared with the rate of growth of the bacteria in the cultures. These results are similar to those reported by Krueger and Northrop (1930) for the growth and lysis of *Staphylococcus aureus* in the presence of an antistaphylococcus phage and by Lin (1934) for the growth and lysis of *Shigella dysenteriae Shiga* in the presence of an antidysentery phage.

The time required for growth to proceed and for lysis to reduce the concentration of bacteria to an arbitrary endpoint that can be readily observed is directly proportional to the initial concentration of phage (temperature, medium, stirring, and age and amount of inoculum maintained constant) over a wide phage concentration range. In the highest dilutions of phage showing lytic activity this proportionality no longer holds, the time required for lysis to reach the arbitrary endpoint increasing markedly.

In figure 2 (*A*, *B*, *C*) the plots of log "total" phage and of log "extracellular" phage against time show a slight lag in phage multiplication during the first 30 to 40 minutes after the cultures are seeded and then the plots of log phage concentrations against time are straight lines almost to the times at which the concentrations of bacteria in the cultures reach maximum values.

During the logarithmic period of phage production the plots of "total" and of "extracellular" phage against time are practically parallel and suggest that a constant ratio is maintained between the concentrations of "total" and "extracellular" phage approximately to the time lysis is initiated. At this time the rate of phage production markedly decreases and the concentrations of "total" and of "extracellular" phage approach a common value.

Within 10 minutes after mixing the phage and bacteria, 70 to 95 per cent of the phage is bound by the cells. During the logarithmic period of phage production the concentration of "extracellular" phage is only 5 to 10 per cent of the "total" phage concentration. This distribution of phage between the cells and the medium is typical of the distribution of a solute between two non-miscible solvents and proceeds in accordance with the equation

$$C_{ec}/C_{ic} = K_d \qquad (1)$$

where C_{ec} represents the concentration of "extracellular" phage, C_{ic} the concentration of "intracellular" phage ("total" minus "extracellular") and K_d the distribution coefficient. Typical results, similar to those reported by Krueger and Northrop (1930) and Krueger (1931), are presented in table 1.

The close agreement between the values of K_d up to the time lysis

is initiated (approximately 110 minutes) suggests the distribution of a chemical compound between the two phases, cells and medium. However, this distribution is not incompatible with the hypothesis that phage may be a living agent, for, if phage is a living parasitic agent one would expect to find the majority of the corpuscles within (or at least bound to) the host cells under favorable conditions; the ratio of free to bound phage increasing only when the rate of lysis exceeds the rate of bacterial multiplication.

The plots of the concentrations of phage and of bacteria against time suggest that the rate of phage multiplication is

TABLE 1

Distribution of phage between the cells and the medium

(Data from figure 2, *B*)

TIME	K_d
minutes	
30	0.089
60	0.083
80	0.093
90	0.083
100	0.076
110	0.125
120	0.224
130	0.338

intimately associated with the rate of growth of the bacteria. This association is more clearly illustrated in table 2 by the changes in the values of K, the multiplication velocity constant, as calculated from the following equations:

$$K_b = 2.303/t \log B/b \tag{2}$$

and

$$K_p = 2.303/t \log P/p \tag{3}$$

where b, or p represents the initial concentration of bacteria, or phage, respectively, and B, or P the concentration at the end of time t.

The values of K_B are fairly constant during the time interval of 30 to 100 minutes. During this period the values of K_P are also quite constant and are considerably higher than the values of K_B. Comparison of the values of K_B and K_P, or of the phage production and bacterial growth curves in figure 2 (*A*, *B*, *C*), suggests that the rate of phage formation is proportional to a power of the rate of bacterial multiplication.

Inspection of figure 2 (*A*, *B*, *C*) indicates that lysis begins (or exceeds the rate of bacterial growth) at the time the log of the

TABLE 2

Multiplication velocity constants of bacterial growth and of increase in phage concentration in a typical culture

(Data from figure 2, *B*)

TIME INTERVAL	K_B	TIME INTERVAL	K_P	
			"Total"	"Extracellular"
minutes		*minutes*		
30–60	0.012	30–60	0.208	0.206
30–80	0.014	30–80	0.207	0.208
30–90	0.011	30–90	0.207	0.206
30–100	0.014	30–100	0.211	0.209
30–110	0.012	30–110	0.199	0.203
110–120	0.034*	30–120	0.199	0.209
110–130	0.067*	30–130	0.183	0.196

* These values represent the rate of lytic destruction of bacteria, B representing the maximum number of bacteria and b the number of survivors after time t; in equation 2.

"total" phage concentration reaches a value 3.1 to 3.3 log units above the log of the bacterial population. When the initial log ratio of phage to bacteria is above this value lysis occurs and little or no growth of bacteria is apparent; when the initial concentration of phage is low, compared with the concentration of bacteria, a longer time is required for lysis to be initiated than in cultures having an initial log ratio of phage to bacteria near the average critical value of 3.2. The latter statement is also illustrated in figure 2 *D* in which numbers of bacteria are plotted against time for different initial concentrations of bacteria, the

initial concentration of phage being 1.0×10^7 phage units per 100 ml.

The ratio of phage to bacteria at the time lysis is initiated (at least under the conditions of these experiments) indicates that 1600 phage units are present per bacterium. Krueger and Northrop (1930), working with staphylococcus cultures and an antistaphylococcus phage, reported that lysis is initiated when 125 phage units per bacterium are present. Since the determination of a phage unit is not highly accurate, the numerical difference between these values has no significance and each of these values would have to be multiplied by certain factors (as yet unknown) to obtain the true ratio of phage to bacteria.

The results presented in figure 2 (*A*, *B*, *C*) also indicate that the major portion of the phage present in the suspension after the completion of lysis is set free from bacteria undergoing lysis. The increase in the concentration of "extracellular" phage per bacterium lysed was calculated from the change in concentration of phage during lysis of 1.0×10^8 bacteria to 0.6×10^8 per milliliter. 1250, 3500 and 8750 phage units were liberated per lysed cell in the cultures containing originally 1×10^5, 1×10^3 and 1×10^1 phage units per milliliter, respectively. This indicates that the same amount of phage is not liberated per lysed cell and that the amount liberated per lysed cell increases with increase in the concentration of phage in the suspension at the time of lysis. In other words the amount of phage liberated per lysed cell is controlled by the distribution of phage between the cell and its environment. However, the rate of lytic destruction of the bacteria appears to be independent of the concentration of phage, since the log bacteria-time curves in figure 2 have the same slope during the period of lysis. During this period the lytic destruction of the cells appears to be logarithmic with time.

When lysis is complete the suspension contains a concentration of phage determined by the phage-bacteria reaction. The final concentrations of phage, in different cultures containing various initial amounts of phage or bacteria, fall within a rather narrow range. This is due to the more rapid rate of increase of phage

than of bacteria and the initiation of lysis when the concentration of phage is approximately 3.2 log units greater than the concentration of bacteria, the maximum bacterial concentration observed in the tests reported in figure 2 lying within the narrow range of log 8.10 to log 8.35 bacteria per milliliter.

SUMMARY AND CONCLUSIONS

The studies reported in this paper on the kinetics of lysis of *Escherichia coli* by an anticoli phage under uniform cultural conditions suggest the following conclusions:

1. The rate of growth of bacteria in the presence of phage, over a wide phage concentration range, does not differ appreciably from that observed in phage-free controls until the time at which lysis is initiated.

2. The time required for bacterial growth to proceed and for lysis to be initiated is directly proportional to the initial concentration of phage, cultural conditions being maintained constant, except in the highest dilutions of phage showing lytic activity.

3. Bacterial multiplication appears to be essential for phage production, both exhibiting short periods of lag during which 90 to 95 per cent of the phage is taken up by the cells.

4. During the logarithmic period of bacterial growth, phage production is also logarithmic but proceeds at a more rapid rate. This suggests that the rate of phage formation is proportional to a power of the rate of bacterial growth. During this logarithmic period of growth, phage is distributed between the cells and the medium in a manner analogous to the distribution of a solute between two non-miscible solvents.

5. Lysis is initiated when the ratio of phage to bacteria exceeds a certain critical value, this ratio being 1600 phage units per bacterium under the conditions of these studies.

6. The major portion of the phage present after lysis is complete is set free from bacteria undergoing lysis, the amount of phage liberated per lysed cell increasing with increase in the concentration of phage at the time lysis is initiated.

7. The lytic destruction of the cells appears to be logarithmic

with time, the rate of lysis being independent of the concentration of phage.

8. The observations of Krueger and Northrop on the kinetics of the reaction between *Staphylococcus aureus* and an antistaphylococcus phage harmonize in general with these observations on the kinetics of the reaction between *Escherichia coli* and an anticoli phage.

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DISSOCIATION AND LACTASE ACTIVITY IN SLOW LACTOSE-FERMENTING BACTERIA OF INTESTINAL ORIGIN

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In 1918 Bronfenbrenner and Davis observed that intestinal bacteria were frequently encountered which failed to show up as lactose fermenters in the routine diagnostic procedure, but which readily did so on subsequent cultivation in media containing lactose in higher concentrations. Studies of the conditions under which the fermentation of lactose by these organisms could be enhanced, with the purpose in view of improving methods for their detection, were reported at that time.

Bacteria exhibiting similar variability in fermentation were early described by Neisser (1906) and Massini (1907), under the name *Bacillus coli-mutabile*. They have been studied more recently by Kriebel (1934), Sandiford (1935), and, with special reference to bacterial variation, by Lewis (1934) and Hall (1935).

In the course of continued studies of the metabolism of slow lactose-fermenting bacteria of the *Escherichia coli* type, we have made some observations which we believe may contribute toward elucidation of metabolic mechanisms which determine lactose fermentation by these organisms.

The cultures used in the present study were isolated from unselected human fecal specimens by plating on lactose agar containing China blue-Rosolic Acid (C.R.) indicator (Bronfenbrenner, 1918). Of six samples examined, slow lactose-fermenting organisms were obtained from one, in which they were the predominating type. Several single colony strains of both the slow-fermenting and typical *E. coli* varieties were transferred

to plain agar slants, and maintained by monthly transplant for use as required.

In order to obtain variants of increased fermenting power from these slow lactose-fermenting cultures, they were subjected to daily serial transfer in a lactose medium as in the method previously used (Bronfenbrenner and Davis, 1918). Some elaborations of technic were introduced to facilitate the study of conditions affecting the change from slow to rapid fermentation, and particularly to determine the parts played by bacterial variation and selection respectively in this change. These will be described.

Duplicate serial transfers of five slow-fermenting strains were made daily in two series of 1 per cent lactose C-R peptone water cultures, one series consisting of open tubes plugged with cotton, the second of Dunham fermentation tubes sealed with paraffin. At the end of each 24 hours, one culture in each series was used to inoculate the subcultures, the duplicate being held in the incubator for daily observation. All sub-cultures from the closed tube series were made from the inner gas measuring tube. At frequent intervals, transfers from the open tube culture series were made into sealed fermentation tubes to observe any changes in fermentation reactions, which were thus always compared under identical conditions. At the same time these cultures were streaked on plates of lactose C-R agar for the purpose of estimating the relative numbers of fermenting variants. The same examination was made of cultures kept without transfer, corresponding to subculture I of each series (table 1). Observed increases in the ability of the cultures to form acid and gas in lactose subculture, were always concurrent with the finding of rapidly fermenting variants on the plates. No changes in fermenting power were seen until the twelfth day in standing cultures, or until the twelfth passage in the transfer series. As the experiment progressed, the fermenting power, and the proportion of fermenting variants present, increased to a maximum sometime about the twentieth day. These changes occurred with the same regularity, but were never as marked, in cultures of the open tube series. The proportion of fermenting colonies

(in per cent of the total colonies) on the plates made from subculture I at 1 day, 10 days, and 20 days, and from subculture XX after 24 hours, for each of the two series of cultures, are compared in table 1.

From the foregoing results it is evident that the change in fermenting power occurring after a prolonged cultivation in lactose medium is due to the appearance of variants and that this change is greater under conditions of partial anaerobiosis.

Furthermore, it appears that while variation occurs after about 12 days, the multiplication of actively fermenting variants thereafter goes on faster than that of non-fermenting variants.

TABLE 1

Differential plate count showing per cent of fermenting colonies in lactose cultures after aging and after serial subcultures

STRAIN	SUBCULTURE I, 0–10 DAYS (PER CENT BLUE COLONIES)		SUBCULTURE I, 20 DAYS (PER CENT BLUE COLONIES		SUBCULTURE XX, 1 DAY (PER CENT BLUE COLONIES)	
	Open tubes	Closed tubes	Open tubes	Closed tubes	Open tubes	Closed tubes
a	0	0	100	100	90	100
b	0	0	5	30	50	100
c	0	0	0	10	10	100
d	0	0	50	30	50	75
e	0	0	10	30	50	100

This appears even more clearly from the following experiment designed to define the growth conditions favoring selection of fermenting variants. Several media made up in fermentation tubes were inoculated in duplicate with pure cultures of fermenting and non-fermenting strains respectively. The media consisted of the following: (1) 1 per cent peptone, 0.5 per cent sodium chloride; (2) 1 per cent lactose added to a synthetic basal medium containing 0.5 per cent ammonium sulfate, 0.5 per cent sodium chloride, and M/20 phosphate buffer of pH 7.0; (3) 3 per cent lactose added to the same base; (4) 1 per cent sodium citrate added to the same base; (5) 1 per cent sodium succinate added to the same base; (6) 1 per cent lactose and 1 per cent sodium succinate combined in the same base; and (7) 1 per cent lactose, 1 per cent peptone and 0.5 per cent sodium chloride.

The relative amounts of growth appearing in 24 hours in the closed and open portions of the tubes for the two variant strains were separately recorded, as shown in table 2.

It is evident from this experiment that conditions of limited oxygen supply are favorable to the selection of fermenting variants, but only when the lactose is accompanied by an additional source of carbon.

The frequent observation of colonial dissociation in lactose-containing media, directed attention to the possibility that these changes might be in some way related to changes in lactose fermenting power. It was particularly of interest to look for

TABLE 2

Relative growth intensity of fermenting and non-fermenting strains in various media as a function of oxygen supply

MEDIUM	OPEN TUBES		CLOSED TUBES	
	Fermenting variant	Non-fermenting parent	Fermenting variant	Non-fermenting parent
(1) Peptone water	+	+	±	±
(2) Lactose 1 per cent (no additional carbon)	++	++	+	±
(3) Lactose 3 per cent (no additional carbon)	+	++	±	±
(4) Citrate medium	+	+	−	−
(5) Succinate medium	+	+	−	−
(6) Lactose-succinate	++	++	+	−
(7) Lactose-peptone	++	++	+	−

possible similarities between the rapidly fermenting variants and true *Escherichia coli*.

Accordingly, colonial variants were isolated from a number of the plates made during the course of the preceding experiments, and maintained in pure culture for subsequent examination. Grossly recognizable forms consisted of smooth and rough, large mucoid, small translucent, and occasional striated and stellate colonial types. Not all colonial variants were found in dissociated cultures of a single strain, although some colonial dissociation accompanied increase in fermenting power in all cases. Both fermenting and non-fermenting variants were obtained in the various colonial forms. Excepting differences in

colonial form, and rate of lactose fermentation, all dissociants appeared to be identical with one another, and with the parent organisms. They were motile, gram-negative rods, tending occasionally toward the coccoid form. No capsules were demonstrated by the method of Hiss, even in the mucoid cultures. They all grew in the citrate medium previously described, and failed to produce indol or H_2S, or to liquefy gelatin. Succinate and lactate were fermented slowly, and formate with explosive gas formation. Glucose, galactose, mannitol, and sucrose were fermented with acid and gas. These organisms, therefore, whether attacking lactose slowly or rapidly, and regardless of their colonial type, differ from typical *E. coli* in growing on citrate and succinate media, and in producing no indol, thus corresponding more closely to *Aerobacter aerogenes*. The failure to find apparent correlation between lactose-fermenting power and colonial form is in accord with the recent experience of Hall (1935).

Of critical importance in considering the relation between rapidly fermenting variants of the slow lactose fermenters and typical *E. coli* species, is the question of their mutability. Hall (1935) and Stewart (1928) as well as other investigators have failed to observe reversion from fermenting variant to non-fermenting parent types. As a result there is a tendency in the literature to refer to these organisms which have "regained" the capacity to ferment lactose actively, as differing in no essential respect from other lactose-fermenting species. On the contrary, in our experiments, by serial cultivation on the above described synthetic medium with sodium succinate as the only source of carbon, reversion was readily effected.

The fermenting variant used was first obtained in single cell culture by repeated plate isolation. A normal *E. coli* strain was used as control. Both cultures were inoculated into the succinate medium, and six successive subcultures were made at two- or three-day intervals. Lactose-fermenting power was measured at intervals by inoculating into lactose fermentation tubes. From the sixth transfer tube, both cultures were then carried through a series of 20 daily subcultures in lactose fer-

mentation tubes. Results are given in table 3. Relative lactose fermenting capacity is indicated by amount of gas measured in linear millimeters in the standard tubes, and presence or absence of acid.

These results show that variants possessing heightened capacity to ferment lactose are by no means stable in this respect as compared to *E. coli* itself. Deprived of lactose-fermenting power by serial cultivation on succinate medium, the culture slowly regains this power during subsequent serial cultivation on lactose, behaving in every respect like the original slow lactose-fermenting strain.

TABLE 3

Loss of lactose-fermenting power on succinate and its recovery in lactose media

	NUMBER OF SERIAL SUB-CULTURE	AMOUNT OF GAS AND ACID IN 5 DAYS ON LACTOSE			
		Fermenting variant		*E. coli* control	
		Gas	Acid	Gas	Acid
On succinate.....	I	45	+	50	+
	IV	0	−	50	+
	VI	0	−	50	+
Subsequently on lactose.....	I	0	−	50	+
	X	10	+	50	+
	XX	35	+	50	+

It was of interest also to inquire to what extent mutability was an invariable characteristic of the slow lactose-fermenting forms. Hall (1935) has defined the criterion of lactose mutation as "repeated isolation of rapid lactose fermenters from a parent type which perpetuates both itself and the mutant indefinitely. . . .," and other investigators (Kriebel, 1934; Stewart, 1928) have concurred in the finding that slow fermenting individuals isolated from the dissociated culture retain their variability. In our experience most strains have done so, but a few single cell strains, after repeated plate-isolation, failed to show any tendency to change in their lactose-fermenting power, although still readily undergoing colonial dissociation like that observed in the parent strain. The strains used were obtained by culturing from a

large number of white colonies appearing on the lactose C-R plate streaked from a dissociated culture of the parent strain, and were purified by repeated plating, and culturing from single colonies. Their mutability was tested by daily serial subculture in lactose broth, and by aging in lactose broth for 20 days. The parent strain was subjected to similar treatment as a control. After the 20-day period, the control strain, the original single cell strains, and organisms from the two series of lactose cultures, were transferred to lactose fermentation tubes for comparison. At the same time, lactose C-R streak plates were made from the lactose cultures. Although colonial variants were recognizable on all of these plates, and both blue and white colonies appeared

TABLE 4

Lactose fermentation stability of a selected strain during dissociation in lactose medium

TREATMENT OF CULTURE	AMOUNT OF GAS AND ACID (FORMED) IN 5 DAYS ON LACTOSE			
	Purified strain		Parent strain control	
	Gas	Acid	Gas	Acid
Untreated original culture	0	−	0	−
20 serial transplants in lactose	0	−	18	+
20 days aging in lactose	0	−	20	+

in most cases, a few strains like the one described in table 4, yielded only white colonies.

In this table are shown the results of fermentation tests with one of these strains, and the control strain, during the course of this experiment. The amount of gas formed in 5 days is given in linear millimeters, and presence or absence of acid as detected by china blue indicator.

From this experiment it is evident that colonial dissociation may occur without accompanying changes in lactose-fermenting power, and that daughter strains may be selected in which the capacity to ferment lactose is more radically and perhaps permanently depressed.

Two series of experiments were now carried out in an effort to determine in what particular the biochemical mechanisms of slow

lactose fermentation differ from those utilized by the actively fermenting strains. These experiments were necessarily concerned therefore with the question whether the lactose-fermenting capacity of the various strains depends exclusively on their ability to hydrolyze lactose to its fermentable monosaccharides, or whether some additional lactose-fermenting mechanism may be involved.

The preliminary experiments consisted of manometric measurements of the respiration of washed, non-proliferating cells in the presence of lactose, and of its constituent monosaccharides, respectively. It was first of all necessary to determine whether, as anticipated, an actual difference could be seen in the capacity of the cellular enzymes of the various strains to ferment lactose, and to exclude the possibility that "secreted" enzymes were primarily concerned. Further, it was reasoned that, if lactose fermentation by these organisms is a quantitative function merely of their lactase activity, then, supposing their monosaccharide-fermenting power to be roughly equal, the observed discrepancy between rates of oxidation of lactose and of monosaccharides respectively would give an inverse measure of lactase content. For the purposes of these measurements, unstandardized bacterial suspensions could be used. Oxygen uptake was measured in the Barcroft apparatus using washed cell suspensions in M/20 phosphate buffer of pH 7.2, containing M/20 lactose. The vessels were provided with side arms permitting the addition of sufficient M/1 solution of the monosaccharide mixture (glucose and galactose) to make the final concentration M/40 with respect to each. Readings of the monometer were made at three successive 10 minute intervals for each substrate, and are expressed directly in the linear values. These values are shown in table 5.

Thus, it was evident that differences in lactose-fermenting power could be attributed to the intracellular enzyme activities of the respective organisms.

Experiments of a second series were designed to give separate comparative measurements of lactose-hydrolyzing, and lactose-fermenting activity of cells of the various cultures. Use was made of the fact that toluol inhibits oxidation processes without destroying hydrolytic function (Karstnom, 1930).

Enzyme preparations consisting of washed cell suspensions of equal turbidity of *E. coli*, a slow lactose-fermenting strain, and a rapidly fermenting variant of the latter, respectively, were divided each into two parts. One of these was shaken up with toluol and left for 2 days, while the other was held as control for the same length of time previous to its use in the experiment.[1] In addition to the cell suspensions, equivalent amounts of supernatant fluid obtained by centrifugation of the fresh cell suspension, and of the same suspension after autolyzing two days under toluol, were tested for lactase activity. They will be referred to as supernatant and autolysate respectively. The test mixtures were made up to contain equal quantities of the

TABLE 5

Stimulation of lactose oxidation by washed cells on addition of monosaccharide

SUBSTRATE	O_2 UPTAKE (CM./10 MIN.)	PER CENT STIMULATION BY MONOSACCHARIDES
For *E. coli*:		
Lactose	1.54; 1.60; 1.60	56
Monosaccharides	2.41; 2.50; 2.52	
For slow-fermenting strain:		
Lactose	0.30; 0.31; 0.30	1,400
Monosaccharides	4.44; 4.57	

preparation to be tested for the enzyme, lactose in 0.5 per cent or glucose in 0.25 per cent concentration, and M/20 phosphate buffer of pH 7.2, when diluted with distilled water to a total volume of 10 cc. Incubation was made at room temperature, and the determinations were carried out after 24 and 48 hours. At the conclusion of each experiment, the mixtures were plated to show absence of contamination. Table 6 lists the mixtures prepared for the examination of each strain, and the changes which occurred during the period of incubation as measured by the Schaffer-Hartmann micro-method for determination of reducing sugar.

[1] Preliminary tests showed that it was necessary to treat the cell suspension with toluol for two days, after which no viable cells remained, and oxidase activity was destroyed.

From these values the amount of *lactose hydrolyzed* was calculated from the increase in glucose value for tube (1) over its control tube (3), multiplied by the lactose copper-reduction value 1.67.

The amount of *lactose fermented* in the absence of toluol in the same period of time was similarly calculated from the decrease in glucose value obtained by comparison of tubes (2) and (3).

Comparison of tubes (4) and (5) showed the amount of *glucose fermented under toluol*, and it was found in each case that toluol

TABLE 6

Copper reduction value calculated as grams per cent of glucose after incubation of test mixture

	STRAINS			
	E. coli		Slow fermenting strain	Fermenting variant strain
	24 hours	48 hours	48 hours	48 hours
1. Lactose, cells, toluol	0.37	0.41	0.26	0.30
2. Lactose, cells	0.20	0.13	0.24	0.16
3. Lactose, boiled cells	0.27	0.27	0.25	0.25
4. Glucose, cells, toluol	0.23	0.22	0.18	0.18
5. Glucose, cells	0		0	0
6. Glucose, boiled cells	0.20	0.22	0.20	0.20
7. Cells alone, toluol	0		0	0
8. Cells alone	0		0	0
9. Lactose, supernatant	0.25	0.26	0.27	0.26
10. Lactose, autolysate	0.27	0.27	0.27	0.28

successfully inhibited monose fermentation. From tubes (5) and (6), giving the amount of *glucose fermented* in the absence of toluol, it was evident that the preparation was capable of active fermentation under these conditions. Examination of tubes (7) and (8) proved that the bacterial suspensions themselves were free from reducing sugars. Tubes containing the autolysate and supernatant fluid, which were included to detect any soluble lactase present, remained unchanged. A summary of the calculated results is given in table 7.

Thus, it is seen that slow lactose-fermenting strains are deficient in the enzyme lactase, and that increase in fermenting

capacity is accompanied by corresponding increase in this enzyme. The lactase of *E. coli* is an intracellular enzyme which is not set free from the protoplasm to any appreciable extent even after the death and autolysis of the cells. There is no indication of a lactose-fermenting mechanism other than preliminary hydrolysis to the constituent monosaccharides.

It should be emphasized, in conclusion, that variations in lactase activity described throughout this paper are merely quantitative and that in no instance under our observation have variants shown any metabolic property which was wholly absent in the parent organism (Diehl, 1919). Thus, the phenomena suggesting specific elaboration of enzymes corresponding to a

TABLE 7

Simultaneous lactose fermentation and lactase activity expressed in grams per cent of sugar changed

ENZYME PREPARATION	TIME	CONTROL (GLUCOSE FERMENTED UNDER TOLUOL)	CONTROL (GLUCOSE FERMENTED)	LACTOSE FERMENTED	LACTOSE HYDROLYZED
	hours				
E. coli cells	24	0	0.20	0.12	0.16
E. coli cells	48	0		0.23	0.23
Slow fermenting strain cells	48	0	0.20	0.02	0.02
Fermenting variant cells	48	0	0.20	0.14	0.12
Original amount of sugar present		0.20	0.20	0.45	0.45

defined substance supplied in the medium in our opinion indicate no more than the realization of a potential, perhaps latent, physiologic capacity of the bacterial species in question.

SUMMARY

1. Slow lactose-fermenting intestinal bacteria of the *Bacillus coli-mutabile* type of Neisser and Massini manifest stable metabolic characters distinct from typical *Escherichia coli* in addition to their lactose mutability.

2. Colonial dissociation invariably occurs concomitantly with metabolic variation in the five strains studied, but no direct correlation between colonial type, and fermentative capacity was observed.

3. It was found possible to induce variation by suitable manipulation both from the slow to rapidly fermenting types, and *vice versa*. Occasional slow-fermenting daughter strains were obtained which appeared stable toward lactose.

4. The fermentation of lactose by the bacteria studied is the function of an intracellular lactase, and its variations are the variations in the activity of this enzyme.

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BACTERIOPHAGE AS RELATED TO THE ROOT NODULE BACTERIA OF ALFALFA[1]

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INTRODUCTION

Bacteriophages virulent for different strains of Rhizobia of leguminous plants have been isolated by various investigators but not much has been learned regarding their significance in the nodules. Laird (1932) established the heterogeneous nature of the Rhizobia with respect to bacteriophage, but his attempt, as well as that of Almon and Wilson (1933), to find a correlation between the effectiveness of different strains in nodulation and sensitiveness to the lytic principle was not successful. Among the theories relative to the means by which atmospheric nitrogen fixed by Rhizobia is made available to the plant, Fred, *et al.*, (1932) have listed the action of bacteriophage, but little or no work has been done in this connection. Demolon and Dunez (1935) isolated a bacteriophage from the soil surrounding the roots of old alfalfa plants and concluded that it interferes with symbiosis between the Rhizobia and the host plant.

Within recent years a decrease in yield of alfalfa, *Medicago sativa*, three to five years after seeding has been noted on irrigated soils in the Yakima and Ellensburg districts in Washington. Marked responses from additions of nitrogen have been obtained in experiments in these districts, pointing to the possibility of the presence of some substance which interferes with normal symbiosis between the nodule bacteria and the alfalfa plant. The object of the work presented here was to determine if Rhizobia bacteri-

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ophage may be responsible for decreases in yield of old stands of alfalfa.

EXPERIMENTAL PROCEDURE

Samples of alfalfa plants, including leaves, stems, and roots, and of soil in contact with, or close to, the tap roots were tested for bacteriophage, using the culture medium of Demolon and Dunez (1935). It contains:

K_2HPO_4	1 gram
$MgSO_4$	0.2 gram
$CaCl_2$	0.1 gram
NaCl	0.1 gram
$FeCl_3$	0.02 gram
Glucose	3 grams
$CaCO_3$	1 gram
Alfalfa root extract	1000 cc.

For the preparation of the root extract 200 grams of finely chopped, fresh, washed alfalfa roots were placed in 1000 cc. of distilled water and boiled gently for two hours. The mixture was kept over night, filtered through cotton, and the filtrate made up to volume. The salts were then added and the reaction of the solution adjusted to pH 7.8.

The soil samples were passed through a 16-mesh sieve and the alfalfa roots and stems and leaves were separated, crushed, and ground in a meat chopper. Portions of 100 grams of soil, root, stem, and leaf tissue were placed in separate flasks containing 500 cc. of the liquid culture medium. The mixtures were kept at 28°C. for 36 to 48 hours, filtered through filter paper, and finally through Berkefeld N candles. Two-cubic centimeter portions of the filtrates were added to 24-hour fluid cultures of *Rhizobium meliloti* (medium 5 of Laird (1932)). After 48 hours of incubation at 28°C., the cultures were filtered through Berkefeld N candles and 2-cc. portions of the filtrate added to fresh 24-hour fluid cultures of *Rh. meliloti*. Clearing of the fluid cultures may be observed following the addition of the initial filtrate or following the second or further serial transfers, depending upon the potency of the lytic principle. At least five serial transfers were made of the filtered extracts of each sample. If no clearing was

effected following the fifth serial transfer, the lytic agent was considered absent and the sample discarded.

The nodules were sterilized with 1:500 $HgCl_2$, thoroughly washed with sterile water, crushed aseptically, and pieces of the crushed nodules placed on streaks made from a suspension of a susceptible strain of *Rh. meliloti* on agar in plates, which were incubated at 28°C.; and lysis, if present, was manifested in 48 to 60 hours by the absence of bacterial growth in the vicinity of the pieces of nodule tissue.

EXPERIMENTAL RESULTS

The extracts of samples of soil, roots, and stems obtained from three-year-old stands of alfalfa in two old fields in the Yakima valley were tested against each of five different strains of laboratory stock cultures of *Rh. meliloti*. The root and stem tissues yielded no lytic principle, but the soil extracts contained a lytic principle that was strongly active against two of the five strains, fairly active against two others, and totally inactive against the fifth after five serial transfers. The roots carried few nodules, but all those tested produced lysis.

Samples of soil and alfalfa obtained in both the Yakima and the Ellensburg districts from fields which have been in alfalfa off and on for many years and also from fields carrying this crop for the first time were used for subsequent experiments. The nodules and the extracts of the samples of soil, alfalfa roots, stems, and leaves were tested against the strain of *Rh. meliloti*, which proved to be most susceptible to lysis in the preliminary tests. The results obtained with the soil extracts and the nodules are summarized in table 1.

As may be noted, bacteriophage was present in several of the soils with old alfalfa stands but not in soils carrying alfalfa less than three years of age. Contrary to the findings of Demolon and Dunez (1935), who isolated bacteriophage from the roots and stems of all alfalfa over one year old, this lytic principle was not found in the roots, stems, or leaves of alfalfa except in one case, in which the roots of three-year-old plants growing on soil containing bacteriophage also showed the presence of the lytic principle.

All the nodules yielded a lytic principle, as in the preliminary tests, and this occurred regardless of the age of the plants carrying the nodules. The presence of the lytic principle was manifested by lysed parts of the streaks on agar plates, as indicated in figure 1.

TABLE 1

Bacteriophage in soils and alfalfa tissues

FIELDS IN ALFALFA OFF AND ON FOR MANY YEARS					FIELDS IN ALFALFA FOR THE FIRST TIME				
Soil sample number	Age of stand	Soil	Nodules	Nodulation	Soil sample number	Age of stand	Soil	Nodules	Nodulation
Yakima District									
	years					*years*			
1	2	−	0	Poor	21	2	−	0	Medium
2	2	−	0	Poor	22	2	−	+	Medium
3	3	+	+	Poor	23	3	+	+	Medium
4	3	+	0	Poor	24	3	−	0	Good
5	3	−	0	Poor					
6	3	+	0	Poor					
7	4	−	0	Medium					
8	4	−	+	Poor					
9	4	+	0	Poor					
10	12	+	0	Poor					
Ellensburg District									
11	1	−	0	Poor	25	2	−	0	Medium
12	1	−	+	Good	26	2	−	0	Good
13	2	−	0	Good	27	3	−	0	Medium
14	3	−	0	Good	28	4	+	0	Poor
15	5	−	0	Medium	29	4	−	0	Medium
16	6	−	0	Poor					
17	6	−	+	Medium					
18	9	+	0	Poor					
19	25	−	0	Good					
20	25	−	0	Medium					

+, bacteriophage present; −, bacteriophage absent; 0, not tested.

These results are in agreement with those of Demolon and Dunez (1935) but at variance with those of Laird (1932), who was able to find bacteriophage only in very young nodules of clover, *Trifolium pratense*, and sweet clover, *Melilotus alba*, in pot cultures or under field conditions.

The results obtained from 29 fields appear to indicate that fields with three- and four-year-old stands of alfalfa are the most common source of bacteriophage, as only two of the eight fields yielding the lytic principle carried alfalfa stands over four years of age,

FIG. 1. LYSIS OF RH. MELILOTI ON SOLID MEDIA BY BACTERIOPHAGE FROM ALFALFA NODULES AND BY BACTERIOPHAGE FILTRATE

A and *B*, streaks of *Rh. meliloti* with pieces of crushed nodule; *C*, untreated "control" streak; and *D*, streak impregnated with one loopful of a homologous bacteriophage.

the stands in these cases being nine and twelve years old. On the whole, nodulation on the plants in the affected fields was poor. All except two of these fields are located in the Yakima district and crop records indicate that for many years five of the affected fields in the Yakima district and one of the two in the Ellensburg

district have carried alfalfa off and on for the major part of that time.

VIRULENCE, PLAQUE FORMATION, AND TRANSMISSIBLE LYSIS

Laird (1932), Almon and Wilson (1933), and Demolon and Dunez (1935) demonstrated that different strains of Rhizobia homologous for specific groups of legume plants are not equally sensitive to the same bacteriophage, and conversely that specific strains of Rhizobia are not equally sensitive to different bacteriophages isolated from the nodules or plant tissues of the same species of legumes. The data in table 2 are in agreement with the first observation of these investigators with respect to the sensitiveness of five strains of *Rh. meliloti* to the action of the lytic principle isolated from the alfalfa field represented by soil 3. Their second contention is supported by the results given in table 3, which show the comparative virulence of the lytic principles isolated from a number of different alfalfa fields against the same strain of *Rh. meliloti*.

A quantitative determination of the virulence of the bacteriophage isolated from soil 3 following eleven serial transfers was attempted. The dilution method of Applemans (1921), as modified by Laird (1932), was employed; and the results are summarized in table 4. It is noted that complete dissolution was effected at a dilution of $1:10^9$ and distinct activity manifested at a dilution of $1:10^{11}$. The virulence of this bacteriophage exceeds that reported by Laird (1932) or by Demolon and Dunez (1935) and is of the same magnitude as a bacteriophage against *Staphylococcus aureus* reported by d'Herelle (1917).

Plaque formation was demonstrated, using the technic of Laird (1932). The lytic agent employed was the one isolated from soil 3, using dilutions $1:10^1$ to $1:10^7$ inclusive following the fifth serial transfer. At the end of 48 hours incubation small, distinctly clear, circular areas appeared on the plates on which dilution $1:10^5$ was spread (see fig. 2). A few plaques were present on the plates receiving dilution $1:10^4$ but not on plates receiving lower dilutions, as the concentration of bacteriophage on these plates was too great and caused complete lysis. Dilutions above

TABLE 2

Action of the same lytic principle isolated from soil 3 on different strains of Rhizobium meliloti

STRAIN OF RH. MELILOTI	ORIGINAL FILTRATE		FIRST SERIAL TRANSFER		SECOND SERIAL TRANSFER		THIRD SERIAL TRANSFER		FOURTH SERIAL TRANSFER	
	24*	48	24	48	24	48	24	48	24	48
A-1	−	−	−	−	−	−	−	−	−	−
A-2	+	++	+++	+++	++++	++++	++++	++++	++++	++++
A-3	−	−	−	−	+	+	+	++	++	++
A-4	−	−	−	+	++	++	+++	+++	++++	++++
A-5	−	−	−	−	+	+	+	++	++	++
Control	−	−	−	−	−	−	−	−	−	−

* Hours in contact with filtrate.

++++, perfectly clear; +++, almost clear; ++, definitely clearer than control; +, very slight clearing; −, as in control.

TABLE 3

Comparative strength of lytic principles from different soils tested against Rhizobium meliloti strain A-2

SOIL SAMPLE NUMBER	ORIGINAL FILTRATE		FIRST SERIAL TRANSFER		SECOND SERIAL TRANSFER		THIRD SERIAL TRANSFER		FOURTH SERIAL TRANSFER		FIFTH SERIAL TRANSFER	
	24*	48	24	48	24	48	24	48	24	48	24	48
3	+	++	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++
4	+	++	+++	+++	+++	+++	++++	++++	++++	++++	++++	++++
6	−	+	++	+++	++	+++	+++	+++	++	++++	++++	++++
9	−	−	+	++	−	++	+	++	++	++	+++	+++
10	−	−	−	−	−	+	+	+++	++++	++++	++++	++++
18	−	−	+	+++	++	++	++	+++	++++	+++		
23	−	+	+++	++	++	+++	++	+++	++++	++++	++++	++++
28	−	−	−	++	+++	+++	++++	+++	+++	+++		
Control	−	−	−	−	−	−	−	−	−	−	−	−

* Hours in contact with filtrate.

++++, perfectly clear; +++, almost clear; ++, definitely clearer than control; +, very slight clearing; −, as in control.

1:10^5 had too small a concentration of the lytic agent, and so the plates were covered with bacterial growth.

TABLE 4

Virulence of Rhizobia bacteriophage isolated from soil 3 carrying three-year-old alfalfa (eleventh serial transfer)

DILUTION	18*	24	48	72	96	120	144	168	192	216
1:10^1	++++	++++	++++	++	+	−	−	−	−	−
1:10^2	++++	++++	++++	+++	+++	++	+	+	−	−
1:10^3	++++	++++	++++	+++	+++	+++	+++	+++	++	+
1:10^4	+++	++++	+++	+++	+++	++	++	−	−	−
1:10^5	++++	++++	++++	++++	+++	+++	+++	+++	++	+
1:10^6	++++	++++	++++	++++	+++	+++	++	++	+	+
1:10^7	+	++++	++++	++++	++	++	−	−	−	−
1:10^8	−	+	++++	++++	+++	++	++	++	−	−
1:10^9	+	++++	++++	++++	+++	+++	+++	++	−	−
1:10^{10}	−	−	+++	+++	+++	+	+	+	−	−
1:10^{11}	−	−	−	−	−	−	+	++	+	+
1:10^{12}	−	−	−	−	−	−	−	−	−	−
1:10^{13}	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−

* Hours in contact with filtrate.

++++, perfectly clear; +++, almost clear; ++, definitely clearer than control; +, very slight clearing; −, as in control.

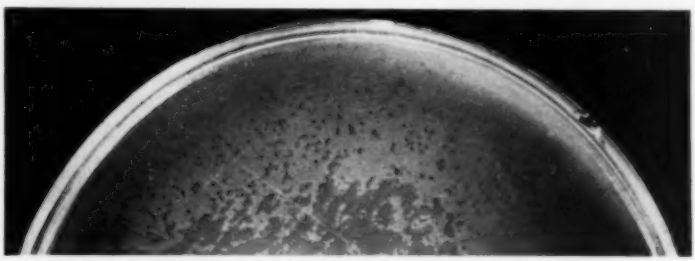

FIG. 2. PLAQUES WITH RH. MELILOTI AFTER 48 HOURS

Inoculum consisted of a 24-hour culture mixed with the lytic agent; dilution, 1:10^5; magnification, 1.25×.

Bacteriophage was removed from a number of distinct plaques and 24-hour fluid cultures of the susceptible strain used throughout the investigation were inoculated with it. Distinct clearing

of the cultures was evident 24 to 48 hours after inoculation, thus indicating that the lytic agent was transmissible.

DISCUSSION

Although the attempts of Laird (1932) and Almon and Wilson (1933) to discover the significance of bacteriophage in the symbiotic relationship of the nodule bacteria and the leguminous plants were not successful, the recent work of Demolon and Dunez (1935) in this regard has been more fruitful. The latter succeeded in isolating bacteriophage from the nodules, roots, and stems of all alfalfa over one year old on "alfalfa sick" soils or what they call "fatigued alfalfa fields" in a number of districts in France, but experienced difficulty or failed in finding it in the roots of alfalfa over three years old or in those of old alfalfa plants devoid of nodules. They also isolated bacteriophage from the soil carrying old stands of alfalfa but not from soils in other crops or carrying young stands of alfalfa. They demonstrated to their own satisfaction that the presence of this lytic agent in the soil or in plant tissues interferes with normal symbiosis of the nodule bacteria and the host plant, and reached essentially the following conclusions: (1) As long as the root system of alfalfa penetrates deeper in the soil, new nodules may be formed and nitrogen fixed even in the presence of bacteriophage. (2) When expansion of the root system is at a maximum, as when the plant attains the age of about two years, the bacteriophage diffuses through the entire root zone and accomplishes the complete disappearance of the Rhizobia, so that the alfalfa then grows like a non-legume. Thereafter, probably in less than one year, the bacteriophage also disappears from the soil.

These sweeping conclusions imply that as the bacteriophage diffuses through the soil complete destruction of all the nodule bacteria takes place eventually, and little or no possibility remains for the occurrence of secondary growth of the more resistant organisms. Complete bacteriolysis by bacteriophage under natural conditions and especially in the soil under field conditions can hardly be expected in the light of present knowledge. This is confirmed by the results of one of Demolon and Dunez's (1935)

experiments in which they used soils from two old alfalfa fields containing very active bacteriophage for *Rh. meliloti*. The soils were placed in pots in quantities of 10 kgm., and sterilized alfalfa seeds were planted. Two months later the roots were examined, and some nodules were found in the presence of active bacteriophage when according to their own conclusions regarding the action of bacteriophage in "alfalfa sick" soils no *Rh. meliloti* should have been present in those soils.

The fact that in the Yakima district both active bacteriophage and nodules were found in two old alfalfa fields with twelve- and nine-year-old stands of alfalfa respectively probably is best explained on the basis that secondary growth of resistant Rhizobia occurs in the presence of active bacteriophage. Therefore, it is unlikely that alfalfa plants which bear the root nodules during the first years of their growth would ever completely lose the benefit of symbiosis of the Rhizobia in later years even though active Rhizobia bacteriophage was present in the soil or plant tissues.

Rhizobia bacteriophage, according to the results of Demolon and Dunez (1935), is easily destroyed by desiccation, insolation, and irrigation. Assuming that this lytic agent is readily removed from the soil by percolating water, as they contend, it is not likely to be eliminated completely from average soils by the addition of five liters of water to 10 kgm. of soil as reported by these investigators. However, the results of their experiment point out the possibility of rapid distribution of Rhizobia bacteriophage in irrigated districts by means of irrigation water, since in irrigation practices drainage and run-off waters of one field are commonly used for the irrigation of other fields along the water line. This may explain the fact that active Rhizobia bacteriophage was isolated from two young fields of alfalfa on virgin soils in the Yakima and Ellensburg districts respectively.

The irregular occurrence of bacteriophage in the areas here investigated, and especially its presence in certain young alfalfa fields on newly cultivated lands where reductions in yields of the crop have not been apparent, as well as in old alfalfa fields where alfalfa has occupied the land off and on for the major part of the

time for many years and where reductions in yields of alfalfa stands older than three years have been observed is not wholly in accord with the results of Demolon and Dunez (1935). It is entirely conceivable, however, that being present in the soil or in the plant tissues the bacteriophage may multiply at the expense of the Rhizobia and this action, therefore will interfere with symbiosis of the Rhizobia and host plant.

The absence of bacteriophage in soils with one- and two-year-old alfalfa stands and its presence in some of the soils carrying alfalfa three years old and over coincides with the observation of the farmers that decreases in yields of alfalfa usually begin to be manifested from three to five years following seeding. The fact that, on the average, nodulation was poor on the plants in the fields from which active bacteriophage was isolated is additional evidence in favor of the activity of the lytic principle in destroying the nodule bacteria and in reducing the beneficial effect of symbiosis.

Unquestionable proof that the Rhizobia bacteriophage is responsible for the reduced yields of old stands of alfalfa in the Yakima and Ellensburg districts is still lacking, though the observed facts point in that direction. From the standpoint of atmospheric nitrogen fixation and the possibility of rapid dissemination of the lytic principle the problem is one of serious concern in irrigated areas where alfalfa constitutes the major crop, and warrants further investigation, which is anticipated.

SUMMARY

A potent lytic principle active against four out of five laboratory stock cultures of *Rhizobium meliloti* was isolated from each of two soils from the Yakima district carrying three-year-old alfalfa stands.

Further investigation of soil and plant samples from 29 alfalfa fields in the Yakima and Ellensburg districts failed to result in the isolation of bacteriophage from soils carrying alfalfa plants less than three years of age. Bacteriophage was isolated from eight soils with an alfalfa stand three years of age or over and from the root tissue of three-year-old plants in one field. The

most common source of bacteriophage was the soil from fields occupied by alfalfa off and on for many years and carrying three- and four-year-old stands.

Indications of the presence of a lytic principle were found in the nodules, regardless of the age of the plants carrying them.

Nodulation on the whole was poor on plants in fields from whose soils lytic principles were isolated.

One of the bacteriophages isolated caused complete dissolution of a 24-hour fluid culture of *Rhizobium meliloti* at a dilution of $1:10^9$ and partial clearing at a dilution of $1:10^{11}$.

The transmissibility of the bacteriophage isolated from one of the soils was demonstrated in fluid cultures and by means of plaques.

The possibility that the bacteriophage is responsible for reduced yields of alfalfa is pointed out; but definite conclusions are reserved pending further investigation.

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THE ACTION OF ALDEHYDES ON CERTAIN CULTURES OF STREPTOCOCCUS LIQUEFACIENS IN MILK[1]

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The data obtained at the Iowa Agricultural Experiment Station on *Streptococcus liquefaciens* indicate that, with certain cultures of this organism, the production of acetylmethylcarbinol in milk can be greatly increased by the addition of acetaldehyde to the milk. In accordance with the many studies of Neuberg and his associates (Neuberg and Hirsch, 1921, and later papers) on yeasts, this relationship suggests that with these cultures acetylmethylcarbinol may be formed by an acetaldehyde condensation, since with added acetaldehyde present there would presumably be more of an opportunity for the condensation than if the only aldehyde available were that produced by the organisms.

Because of the great increases in acetylmethylcarbinol production that were obtained when acetaldehyde was added to milk cultures of certain *S. liquefaciens* strains, the effect of other aldehydes was studied. A point of particular interest was whether or not the addition of aldehydes higher than acetaldehyde would result in the formation of compounds which, on oxidation with ferric chloride, would yield homologues of diacetyl. For example, the addition of propionaldehyde might be expected to yield acetylpropionyl ($CH_3—\overset{\overset{O}{\|}}{C}—\overset{\overset{O}{\|}}{C}—CH_2—CH_3$) if it were condensed with acetaldehyde produced by the organism and the condensation product oxidized to the corresponding diketone.

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METHODS

The cultures of *S. liquefaciens* employed were selected on the basis of their ability to produce relatively large amounts of acetylmethylcarbinol at 21°C.; at 37°C. the production is much lower.

For quantitative determinations, the cultures (with or without aldehyde) were steam-distilled with ferric chloride added and the distillates treated with hydroxylamine hydrochloride, sodium acetate, and nickel chloride, according to the method used by Michaelian and Hammer (1935). The *S. liquefaciens* cultures yielded no diketone when distilled without the addition of ferric chloride, so that the values obtained represent diketone (diacetyl, a homologue, or a mixture) formed through an oxidation.

Quantities of precipitate (nickel dimethylglyoximate, a homologue, or a mixture) were prepared by steam-distilling relatively large amounts of culture, after adding ferric chloride, and treating the distillate with the reagents needed to yield the salt desired; 200 ml. of 40 per cent ferric chloride (40 grams made up to 100 ml. with distilled water) were used for 1,000 ml. of culture, and the other reagents were employed in amounts sufficient to insure complete precipitation of any diketone present. Each precipitate was washed repeatedly with distilled water by decantation and then collected, with suction, on a crucible having a fused-in sintered filter disc; the precipitate was also thoroughly washed on the crucible. The salt was removed from the crucible and dried at a temperature slightly above 100°C.

The nickel contents of the salts were determined by decomposing, with sulfuric and nitric acids, thoroughly dried aliquots and precipitating the nickel as dimethylglyoximate which was collected and weighed.

The acetaldehyde, propionaldehyde, n-butyraldehyde, and n-valeraldehyde were obtained from the Eastman Kodak Company, the formaldehyde was a Merck preparation, and the furfuraldehyde[2] was freshly distilled prior to use.

[2] Dr. Henry Gilman, of the Chemistry Department, kindly supplied the furfuraldehyde and also contributed to the work in various ways.

EXPERIMENTAL

The effect of acetaldehyde or propionaldehyde on the production, in a milk culture of *S. liquefaciens*, of a compound which yielded nickel dimethylglyoximate or a homologue when the culture was steam-distilled with ferric chloride added, and the distillate then treated with the necessary reagents is shown in table 1; the aldehydes were added to the cultures at the time of inoculation. With organism 34, three of the acetaldehyde additions gave large increases in the yield of nickel salt which differed from each other only slightly; the largest addition gave a decrease in the yield which was presumably due to an inhibition of the

TABLE 1

Effects of acetaldehyde and propionaldehyde on the production, in milk cultures of S. liquefaciens, of a compound which yielded nickel dimethylglyoximate or a homologue when the culture was steam distilled with ferric chloride added and the distillate treated with the proper reagents

Aldehydes added at time of inoculation. Incubation 7 days at 21°C.

	ALDEHYDE PER 300 ML. CULTURE	Ni SALT PER 200 GRAMS CULTURE DISTILLED WITH $FeCl_3$
	ml.	*mgms.*
Organism 34:		
Milk alone		39.9
Milk alone		41.2
Milk + acetaldehyde	0.05	65.1
Milk + acetaldehyde	0.10	63.5
Milk + acetaldehyde	0.20	69.5
Milk + acetaldehyde	0.30	32.1*
Milk + propionaldehyde	0.05	50.1
Milk + propionaldehyde	0.10	56.2
Milk + propionaldehyde	0.20	55.5
Milk + propionaldehyde	0.30	55.2
Organism 65:		
Milk alone		36.4
Milk + acetaldehyde	0.05	57.2
Milk + acetaldehyde	0.20	72.2
Milk + acetaldehyde	0.40	4.6*
Milk + propionaldehyde	0.05	43.5
Milk + propionaldehyde	0.20	50.7

* Not curdled at time of distillation.

organism. All four additions of propionaldehyde gave increases in the yield of salt. These differed only slightly and were smaller than the increases obtained with three of the acetaldehyde addi-

TABLE 2

Effects of various aldehydes on the production, in milk cultures of S. liquefaciens, of a compound which yielded nickel dimethylglyoximate or a homologue when the culture was steam distilled with ferric chloride added and the distillate treated with the proper reagents

Aldehydes added about 24 hours after inoculation. Incubation 7 days at 21°C.

	ALDEHYDE PER 300 ML. CULTURE	Ni SALT PER 200 GRAMS CULTURE DISTILLED WITH $FeCl_3$
	ml.	*mgm.*
Organism 34:		
Milk alone		64.6
Milk alone		67.9
Milk + acetaldehyde	0.10	102.0
Milk + acetaldehyde	0.15	108.8
Milk + propionaldehyde	0.10	98.1
Milk + propionaldehyde	0.20	99.6
Milk + n-butyraldehyde	0.10	79.8
Milk + n-butyraldehyde	0.20	89.5
Milk + n-valeraldehyde	0.10	104.1
Milk + n-valeraldehyde	0.20	102.8
Milk + n-valeraldehyde	0.40	111.0
Organism 65:		
Milk alone		63.6
Milk alone		58.4
Milk + acetaldehyde	0.10	97.0
Milk + acetaldehyde	0.20	96.3
Milk + propionaldehyde	0.10	86.2
Milk + propionaldehyde	0.20	89.1
Milk + n-butyraldehyde	0.10	73.1
Milk + n-butyraldehyde	0.20	89.9
Milk + n-butyraldehyde	0.40	90.2
Milk + n-valeraldehyde	0.20	92.7
Milk + n-valeraldehyde	0.40	107.6

tions. Organism 65 also gave larger yields of salt when acetaldehyde or propionaldehyde was added to a culture than when no aldehyde was used, although with the largest addition of acetaldehyde there was a striking decrease in the yield. With 0.2 ml.

of acetaldehyde per 300 ml. of culture, the yield of salt was about twice the yield obtained from milk alone.

Observations on the time of coagulation of the various cultures used in securing the results given in table 1 indicated that the aldehydes commonly inhibited the organisms. In the extreme cases the cultures had not curdled at the time of distillation.

Table 2 gives additional data on the effects of aldehydes; the aldehydes were added about 24 hours after inoculation. With organism 34, all four of the aldehydes in the quantities that were used greatly increased the yield of nickel salt. Each of the aldehydes gave essentially the same increase with one addition as with another, and there were no great differences in the effects of the various aldehydes, although the increases were somewhat smaller with n-butyraldehyde than with the others. With organism 65, the four aldehydes had essentially the same effects as with organism 34. It should be noted that in the trials reported in table 2 the two organisms gave considerably larger yields of nickel salt in milk alone and in milk with added aldehyde than in the trials reported in table 1.

The nickel contents of the salts obtained when various aldehydes were added to milk cultures of *S. liquefaciens*, the cultures later steam-distilled with ferric chloride added, and the distillates then treated with the usual reagents are shown in table 3. The data on the milligrams of nickel salt obtained quantitatively per 200 grams of culture show that with each organism there was an increase in the yield of salt when an aldehyde was added to the milk culture, and the same relationship is also evident from the weights of the large preparations of salts. The nickel contents of the various salts differed only slightly, whether the salt was prepared from a culture in milk alone, in milk with added acetaldehyde, or in milk to which a higher aldehyde (n-butyraldehyde or n-valeraldehyde) had been added. These nickel contents were in general agreement with the nickel content of nickel dimethylglyoximate[3] rather than with one of the homologues.

[3] The theoretical nickel content of nickel dimethylglyoximate = 20.32 per cent; of nickel ethylmethylglyoximate = 18.52 per cent; of nickel diethylglyoximate = 17.02 per cent. The determined nickel content of a salt made from a commercial diacetyl preparation was 20.30 per cent.

TABLE 3

Effects of various aldehydes on the nickel content of salts obtained from milk cultures of S. liquefaciens, when the cultures were steam distilled with ferric chloride added, and the distillates treated with reagents to yield nickel dimethylglyoximate or a homologue

Incubation 8 or 9 days at 21°C.

	TIME TO COAGULATE AFTER INOCULATION	Ni SALT PER 200 GRAMS CULTURE DISTILLED WITH $FeCl_3$	SALT SECURED FROM 5,800 ML. CULTURE			
			Weight	Per cent Ni		
				a	b	Average
	days	*mgm.*				
Organism 6:						
6,000 ml. milk inoculated; no aldehyde added	2.0	62.2	1.4376	20.29	20.31	20.30
6,000 ml. milk inoculated; 3 ml. acetaldehyde added 27 hours later	2.0	89.7	1.7089	20.28	20.29	20.285
6,000 ml. milk inoculated; 6 ml. n-valeraldehyde added 27 hours later	2.0	83.4	1.8302	20.07	20.12	20.095
Organism 34:						
6,000 ml. milk inoculated; no aldehyde added	1.0	51.1	1.0212	20.25	20.37	20.31
6,000 ml. milk inoculated; 3 ml. acetaldehyde added 12 hours later	2.5	89.5	1.3385	20.23	20.39	20.31
6,000 ml. milk inoculated; 8 ml. n-valeraldehyde added 12 hours later	3.5	97.4	2.1627	20.08	19.88	19.98
Organism 65:						
6,000 ml. milk inoculated; no aldehyde added	2.0	48.1	1.0696	20.29	20.31	20.30
6,000 ml. milk inoculated; 3 ml. acetaldehyde added 24 hours later	2.0	64.1	1.4936	20.25	20.22	20.235
6,000 ml. milk inoculated; 4 ml. n-butyraldehyde added 24 hours later	2.0	55.0	1.2468	20.27	20.14	20.205

However, a slightly lower nickel content was obtained with the n-valeraldehyde in the case of the two organisms with which it was used. Additional nickel determinations on the salts confirmed the results, so that it appears there was a tendency to form

TABLE 4

Effects of formaldehyde and furfuraldehyde on the production, in milk cultures of S. liquefaciens, of a compound which yielded nickel dimethylglyoximate or a homologue when the culture was steam distilled with ferric chloride added and the distillate treated with the proper reagents

Incubation 7 days at 21°C.

	ALDEHYDE PER 300 ML. CULTURE	Ni SALT PER 200 GRAMS CULTURE DISTILLED WITH $FeCl_3$	TIME OF ADDING ALDEHYDE
	ml.	*mgm.*	
Organism 3:			
Milk alone		30.9	
Milk alone		33.2	
Milk + dilute formaldehyde*	0.05	32.5	When inoculated
Milk + dilute formaldehyde	0.10	21.4	When inoculated
Milk + dilute formaldehyde	0.30	0.0	When inoculated
Milk + dilute formaldehyde	0.50	0.0	When inoculated
Organism 34:			
Milk alone		44.2	
Milk alone		44.4	
Milk + dilute formaldehyde*	0.05	43.4	When inoculated
Milk + dilute formaldehyde	0.10	18.3	When inoculated
Milk + dilute formaldehyde	0.50	0.0	When inoculated
Milk + dilute formaldehyde	1.00	0.0	When inoculated
Milk + acetaldehyde	0.05	67.1	When inoculated
Milk alone		67.9	
Milk alone		64.6	
Milk + furfuraldehyde	0.05	66.9	1 day after inoculation
Milk alone		55.4	
Milk alone		57.7	
Milk + furfuraldehyde	0.05	58.9	When inoculated
Milk + furfuraldehyde	0.20	47.4	When inoculated
Organism 65:			
Milk alone		34.0	
Milk alone		34.0	
Milk + dilute formaldehyde*	0.05	33.2	When inoculated
Milk + dilute formaldehyde	0.10	22.4	When inoculated
Milk + dilute formaldehyde	0.30	0.0	When inoculated
Milk + dilute formaldehyde	0.50	0.0	When inoculated
Milk + acetaldehyde	0.10	55.8	When inoculated

* Dilute formaldehyde = 1 ml. of formalin made up to 10 ml. with sterile water.

TABLE 4—*Continued*

	ALDEHYDE PER 300 ML. CULTURE	Ni SALT PER 200 GRAMS CULTURE DISTILLED WITH $FeCl_3$	TIME OF ADDING ALDEHYDE
	ml.	*mgm.*	
Organism 65—*Continued*			
Milk alone		63.6	
Milk alone		58.4	
Milk + furfuraldehyde	0.05	62.9	1 day after inoculation
Milk + furfuraldehyde	0.10	56.0	
Milk alone		52.7	
Milk alone		57.5	
Milk + furfuraldehyde	0.05	57.9	When inoculated
Milk + furfuraldehyde	0.10	50.3	

a small amount of a higher homologue of diacetyl under the experimental conditions provided, although this tendency must have been slight to influence the nickel values so little.

Since the various aldehydes which increased the yield of nickel dimethylglyoximate seemed to inhibit the growth of the organisms in milk, the effect of formaldehyde or furfuraldehyde on the yield of salt was studied. The data obtained are given in table 4. With each of the aldehydes small additions to the milk cultures had no effect on the yield, while larger additions decreased the yield; with some of the quantities of formaldehyde used, no nickel salt was obtained, due undoubtedly to the great interference with the growth of the organisms.

CONCLUSIONS

With certain cultures of *Streptococcus liquefaciens* which gave a greater yield of acetylmethylcarbinol when acetaldehyde was added to the milk in which the cultures were grown than when no acetaldehyde was added, such aldehydes as propionaldehyde, n-butyraldehyde, or n-valeraldehyde also gave an increased yield of acetylmethylcarbinol. The expected production, in considerable amounts, of homologues of diacetyl when cultures to which n-butyraldehyde or n-valeraldehyde had been added were steam-

distilled in the presence of ferric chloride did not occur, and the nickel contents of the derivatives obtained from the distillates with hydroxylamine hydrochloride and nickel chloride (in the presence of sodium acetate) were essentially the same as the nickel contents of derivatives obtained from cultures in milk alone or in milk containing acetaldehyde. However, in the two trials with n-valeraldehyde, the nickel contents of the salts were slightly lower than the theoretical for nickel dimethylglyoximate, which suggests that there was a tendency to form a small amount of a higher homologue of diacetyl under the experimental conditions provided. The addition of formaldehyde or furfuraldehyde to the milk in which the organisms were grown, gave no increase in the production of a compound yielding a nickel salt with the usual analytical procedure.

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EFFECT OF THE CHEMICAL CONSTITUTION OF SOAPS UPON THEIR GERMICIDAL PROPERTIES[1]

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Studies on the properties of soaps are of importance because of their common use as detergents, in the preparation of vaccines, in the treatment of disease, and because of their natural occurrence in the animal body where they may have some effect in determining the resistance of the animal organism to infection.

Reichenbach (1908) studied the action of soaps on *Escherichia coli* and found that potassium stearate, palmitate, and myristate were effective in killing this organism, while the oleate, erucate, and linoleate were inactive.

Walker (1924–1926) showed that sodium and potassium soaps of the same acid did not vary greatly in their germicidal action. The lower members of the series such as the butyrate, valerate, caprylate, and caproate, had little or no germicidal effect. He found the pneumococcus to be very susceptible to laurates, oleates linoleates, and linolenates. Streptococci were killed much like the pneumococci but at higher concentrations of the soap. *Escherichia coli*, *Eberthella typhi*, *Shigella paradysenteriae*, *Salmonella paratyphi* were killed by moderate concentrations (N/20 to N/40) of the saturated soaps (laurates to stearates) but were very resistant to the unsaturated soaps.

Eggerth (1926–1931) studied the germicidal action of alpha substituted soaps. He found that the soaps of the alpha bromo fatty acids were usually more germicidal than the unsubstituted. The alpha hydroxy soaps exhibited a high germicidal activity

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toward certain microörganisms. The effect of the hydroxyl in the alpha position was to increase the selective germicidal action of saturated soaps and diminish that of the unsaturated. Certain of the alpha mercapto and disulfo soaps proved to be powerful germicides.

Recently there has been considerable interest manifested in the germicidal properties of sodium ricinoleate. Larson (1921) found that pneumococci and streptococci would not grow in the presence of even small amounts of sodium ricinoleate. Larson and Nelson (1925) reported that pneumococci instantly lost their pathogenicity on treatment with castor oil soap of a final dilution of 0.1 per cent. Scarlet fever streptococci lost their power to grow after 5 minutes in 0.5 per cent sodium ricinoleate.

Miller and Castles (1931) found that sodium ricinoleate inhibited the growth of gonococci on artificial media in dilutions of 1:20,000.

Violle (1933) studied the effect of a 1:1000 solution of castor oil soap on many kinds of bacteria. The common and pathogenic bacteria of the intestinal tract were unaffected. Streptococci were killed, but staphylococci were not.

Barnes and Clark (1934) from their experiments determined that 0.004 per cent sodium ricinoleate and 0.0004 per cent sodium oleate were approximately the minimal pneumococcidal concentrations of the soaps against three types of pneumococci.

Kolmer, Rule, and Madden (1934) reported that whereas a 20 per cent solution of sodium ricinoleate was completely bactericidal for *Staphylococcus aureus* in an exposure of 5 minutes, yet a 10 per cent solution was not completely bactericidal in exposures as long as 1 hour when tested according to the Reddish method.

Hettche (1934) found that the unsaturated soaps such as the oleate, linoleate, and linolenate, were germicidal toward the staphylococcus but not toward the colon bacillus.

EXPERIMENTAL

The soaps and closely related compounds studied in this investigation, along with their structural formulas, melting points or iodine numbers, and sources or methods of preparation are given in table 1.

The technique used in the experimental work was planned with the idea in mind that these soaps might have some therapeutic value. Sodium ricinoleate has been studied quite extensively in this direction by Kolmer, Rule, and Madden (1934) and others, as has sodium chaulmoograte and similar soaps on the acidfast bacteria by Adams and his co-workers (1932).

Each of the soaps has been tested for its germicidal effectiveness in 1 per cent solution, using *Staphylococcus aureus* (F. D. A. strain) and *Escherichia coli-communis* (U. of M. strain). The least amounts necessary to kill *Diplococcus pneumoniae* (type I) and *Streptococcus lactis* have been determined in two different manners. In all this work, the soaps and solutions have been adjusted as closely as possible to a pH of 8.0, as determined by the glass electrode method, except where otherwise stated.[2]

The soap solutions were in general prepared by dissolving weighed quantities of the fatty acids in a slight excess of dilute sodium hydroxide to make a 2 per cent solution, which was then adjusted to the proper pH by the addition of dilute hydrochloric acid. The solutions were sterilized by heating in boiling water. Dilutions were made with sterile distilled water. The germicidal tests using *Diplococcus pneumoniae* (type I) and *Streptococcus lactis* were performed with the soap solution containing N/30 phosphate buffer of pH 8.0.

The organisms used in performing the tests were the Insecticide Board strain of *Staphylococcus aureus*, University of Minnesota strain of *Escherichia coli-communis*, University of Minnesota strain of *Diplococcus pneumoniae*, type I, pathogenic to mice and rabbits, and *Streptococcus lactis* which was isolated from milk and grew well at 37°C. The *Streptococcus lactis* had a tendency to lose its ability to ferment lactose without any change in its other fermentation reactions or in its resistance to phenol or soaps. Once it had lost its ability to ferment lactose, it did not regain this attribute under any of the conditions to which it was subjected. The test cultures were grown in nutrient broth, transferred every 24 hours, and incubated at 37°C. The nutrient broth was prepared in accordance with the recommendations

[2] The glass electrode instrument was kindly furnished by Dr. Allen Hemingway (1935) of the Department of Physiological Chemistry, University of Minnesota.

TABLE 1

Structural formulas of compounds

SUBSTANCE	FORMULA	MELTING POINT	IODINE NUMBER	SOURCE OR METHOD OF PREPARATION
Stearic acid	$CH_3(CH_2)_{16}COOH$	69–70°		Eastman Kodak Company
Palmitic acid	$CH_3(CH_2)_{14}COOH$	61–62°		Eastman Kodak Company
Myristic acid	$CH_3(CH_2)_{12}COOH$	52–53°		Eastman Kodak Company
Lauric acid	$CH_3(CH_2)_{10}COOH$	42–43°		Eastman Kodak Company
Oleic acid	$HC(CH_2)_7COOH$ ‖ $HC(CH_2)_7CH_3$			Eastman Kodak Company
Linoleic acid	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$		175	Reduction tetrabromide (M. P., 113–114°) from cottonseed oil
Linolenic acid	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$		268	Reduction hexabromide (M. P., 179–180°) from linseed oil
Clupanodonic acid	$CH_3(CH_2)_2C{\equiv}C(CH_2)_4CH{=}CH(CH_2)_2CH{=}CHCH_2CH{=}CH(CH_2)_2COOH$		312	Reduction octobromide (M. P., greater than 200) from cod liver oil
Alpha elaeostearic acid	$CH_3(CH_2)_3CH{=}CHCH{=}CHCH{=}CH(CH_2)_7COOH$	47–48°		Crystallization of fatty acids of tung oil
Beta elaeostearic acid	$CH_3(CH_2)_3CH{=}CHCH{=}CHCH{=}CH(CH_2)_7COOH$	71–72°		Action of crystal of iodine on alpha elaeostearic acid
Undecylenic acid	$CH_2{=}CH(CH_2)_8COOH$			Vacuum distillation of castor oil
Ricinoleic acid	$HC(CH_2)_7COOH$ ‖ $HCCH_2CHOH(CH_2)_5CH_3$		84	Purification of castor oil fatty acid

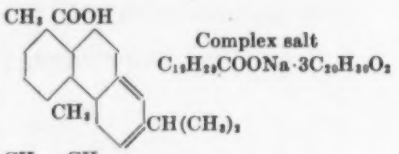
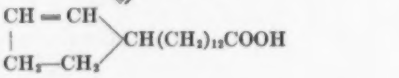
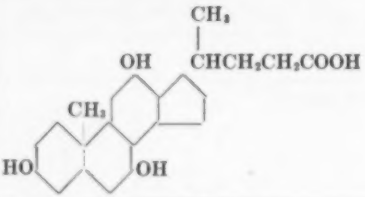
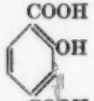
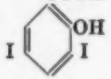
Ricinelaidic acid	$HC(CH_2)_7COOH$ ‖ $CH_3(CH_2)_5CHOHCH_2CH$	52–53°		Action of HNO_2 on ricinoleic acid
Ricinstearolic acid	$C(CH_2)_7COOH$ ‖ $C\,CH_2CHOH(CH_2)_5CH_3$	51–52°		Action of alcoholic KOH on ricinoleic dibromide
Abietic acid	 Complex salt $C_{19}H_{29}COONa \cdot 3C_{20}H_{30}O_2$	166–167°		Purification of rosin
Chaulmoogric acid		69–70°		Eastman Kodak Company
Trihydroxystearic acid	$HCOH(CH_2)_7COOH$ ‖ $HCOHCH_2CHOH(CH_2)_5CH_3$	140–141°		Alkaline oxidation of sodium ricinoleate
Sativic acid	$CH_3(CH_2)_4CHOHCHOHCH_2CHOHCHOH(CH_2)_7COOH$	172–173°		Alkaline oxidation of linoleic acid
Calcium gluconate	$[CH_2OH(CHOH)_4COO]_2Ca$			Eastman Kodak Company
Cholic acid				Eastman Kodak Company Sodium cholate

TABLE 1—*Concluded*

SUBSTANCE	FORMULA	MELTING POINT	IODINE NUMBER	SOURCE OR METHOD OF PREPARATION
Dehydrocholic acid	—OH s in cholic acid replaced by =O			Riedl de Haen Company
Sodium taurocholate	$CHCH_3CH_2COOH$ in cholic acid replaced by $CHCH_2CH_2CONHCH_2CH_2SO_3Na$			Pfanstiehl Company
Sodium glycocholate	$CHCH_3CH_2COOH$ in cholic acid replaced by $CHCH_2CH_2CONHCH_2COONa$			Pfanstiehl Company
Salicylic acid		155–157°		Eastman Kodak Company
3,5 diiodosalicylic acid		235–236°		Eastman Kodak Company
Sodium lauryl sulfate	$CH_3(CH_2)_{10}CHOSO_3Na$			Extraction of Gardinol WA with C_2H_5OH
Sodium oleyl sulfate	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CHOSO_3Na$			Same with Gardinol CA Gardinols obtained from Dupont de Nemours Company

for the Reddish tests, and the nutrient agar used for the final plates was of the same composition, except that 1.5 per cent agar was added.

Plate counts were used in the case of *Staphylococcus aureus* and *Escherichia coli* because of the high resistance of these two organisms to soaps. Plate counts aid in revealing slight bactericidal action, and help to overcome the errors of random sampling, as shown by Cade and Halvorson (1934). Phenol controls were run with each of the tests in order to determine whether the organism had a constant resistance equal to that recommended by Reddish (1928).

DISCUSSION

In this investigation, the germicidal action of 27 soaps and other sodium salts of carboxylic acids and sulfate esters has been tested on four different organisms. Carboxylic acids containing in their structural makeup aromatic rings, iodine, 1 to 4 double bonds, 1 to 5 hydroxyl and cyclopentenyl rings, as well as two sulfate esters were included among those tested. Most of the fatty acids studied in this series occur naturally in mixtures containing several individuals. A few, such as ricinelaidic, ricinstearolic, trihydroxy stearic, sativic and beta elaeostearic, have been synthesized in order to determine how changes brought about in the molecule alter the effect of the soap on microorganisms. The salicylates have been included because of their wide use in acute rheumatic fever. The sulfate esters are new commercial products being sold under the trade names of "Dreft," "Gardinol," and "Brilliant Avirol." These esters are excellent surface tension depressants whose chief feature is their solubility in the presence of calcium, magnesium, hydrogen, or hydroxyl ions.

A comparison of the pneumococcidal properties of the saturated soaps, laurate, myristate, palmitate, and stearate, shows that there is a maximum in this property with the soap containing fourteen carbon atoms. The low activity of the sodium stearate is probably due to its low solubility. The 1 per cent solution of this soap at 37°C. was a semi-rigid gel. The

TABLE 2

Concentrations of chemical sterilizing in 10 but not in 5 minutes

SUBSTANCE	DIPLOCOCCUS PNEUMONIAE	STREPTOCOCCUS LACTIS
	per cent	*per cent*
Germicide:		
Phenol, 20°	1 (1–100)	1.25 (1– 80)
Phenol, 37°	0.588 (1–170)	0.833 (1–120)
Saturated soaps:		
Sodium stearate	1.0	1.0
Sodium palmitate	0.1	1.0
Sodium myristate	0.01	0.1
Sodium laurate	0.04	0.1
Unsaturated soaps:		
Sodium oleate	0.004	0.1
Sodium linoleate	0.005	0.02
Sodium linolenate	0.005	0.02
Sodium clupanodonate	0.005	0.02
Sodium alpha elaeostearate	0.03	>5.0
Sodium beta elaeostearate	0.4	>2.0
Sodium undecylenate	0.2	0.6
Unsaturated hydroxylated soaps:		
Sodium ricinoleate	0.025	0.06
Sodium ricinelaidate	0.05	0.6
Sodium ricinstearolate	0.07	2.0
Unsaturated alycyclic soaps:		
Sodium abietate	0.06	0.2
Sodium chaulmoograte	0.04	0.5
Hydroxylated salts:		
Sodium trihydroxy stearate	>2.0	>2.0
Sodium sativate	0.05	1.0
Calcium gluconate	>10.0	>10.0
Bile salts:		
Sodium cholate	7.0	>10.0
Sodium dehydrocholate	>4.0	>4.0
Sodium taurocholate	7.0	>10.0
Sodium glycocholate	10.0	>10.0
Aromatic carboxylic acid salts:		
Sodium salicylate	5.0	>10.0
Sodium diiodosalicylate	0.1	0.3
Sulfate ester salts:		
Sodium lauryl sulfate	0.01	0.02
Sodium oleyl sulfate	0.01	0.02

TABLE 3

Effect upon Staphylococcus aureus (F. D. A. strain)

SUBSTANCE	CONCENTRATION	PLATE COUNTS		
		5 minutes	10 minutes	15 minutes
	per cent			
Germicide:				
Phenol	1,11 (1–90)	300	50	10
	1 (1–100)	600	200	50
Saturated soaps:				
Sodium stearate	1	10,000+	10,000+	10,000+
Sodium palmitate	1	10,000+	10,000+	10,000+
Sodium myristate	1	10,000+	10,000+	10,000+
Sodium laurate	1	10,000	8,000	6,000
Sodium laurate (pH 8.8)	1	9,000	7,000	4,000
Unsaturated soaps:				
Sodium oleate	1	10,000+	10,000+	10,000+
Sodium linoleate	1	10,000+	10,000+	10,000+
Sodium linolenate	1	10,000+	10,000+	10,000+
Sodium clupanodonate	1	10,000+	10,000+	10,000+
Sodium alpha elaeostearate	1	10,000+	10,000+	10,000+
Sodium beta elaeostearate	1	10,000+	10,000+	10,000+
Sodium undecylenate	1	600	250	100
Unsaturated hydroxylated soaps:				
Sodium ricinoleate	1	10,000+	10,000+	10,000+
Sodium ricinelaidate	1	10,000+	10,000+	10,000+
Sodium ricinstearolate	1	10,000+	10,000+	10,000+
Unsaturated alicyclic soaps:				
Sodium abietate (pH 9.0)	1	5	1	0
Sodium abietate (pH 8.7)	1	150	70	30
Sodium chaulmoograte	1	10,000+	10,000+	10,000+
Hydroxylated salts:				
Sodium trihydroxy stearate	1	10,000+	10,000+	10,000+
Sodium sativate	1	10,000+	10,000+	10,000+
Calcium gluconate	1	10,000+	10,000+	10,000+
Bile salts:				
Sodium cholate	1	10,000+	10,000+	10,000+
Sodium dehydrocholate	1	10,000+	10,000+	10,000+
Sodium taurocholate	1	10,000+	10,000+	10,000+
Sodium glycocholate	1	10,000+	10,000+	10,000+

TABLE 3—*Concluded*

SUBSTANCE	CONCENTRATION	PLATE COUNTS		
		5 minutes	10 minutes	15 minutes
	per cent			
Aromatic carboxylic acid salts:				
Sodium salicylate	1	10,000+	10,000+	10,000+
Sodium diiodosalicylate	1	5,000	1,500	500
Sulfate esters:				
Sodium lauryl sulfate	1	10,000+	10,000+	10,000+
Sodium oleyl sulfate	1	10,000+	10,000+	10,000+

palmitate soap shows an increased destructive action on the pneumococcus, most probably because of its increased solubility. The myristate exhibits the most germicidal activity against the pneumococcus of these saturated soaps, with the laurate soap second in this property.

The presence of unsaturated groupings in the molecule in some cases alters tremendously the effect of the soap upon pneumococci. The sodium oleate which comprises approximately 50 per cent of ordinary tallow soap is more than 100 times as effective as either sodium stearate or phenol in destroying the reproductive ability of the pneumococci. The addition of one, two, or three more double bonds as in the linoleate, linolenate, and clupanodonate soaps, respectively, causes no change in this property. However, the two soaps, sodium alpha and beta elaeostearates, although isomeric with sodium linolenate, are relatively ineffective as pneumococcides. The former of these two soaps, which occurs naturally in tung oil, is as soluble as the sodium linolenate and has much the same physical and chemical properties. Yet a mere change in position of the unsaturated linkages markedly altered the property of destroying this organism.

The three unsaturated soaps containing hydroxyl groups, as well as the two alicyclic unsaturated soaps, show pneumococcidal properties considerably less than those of sodium oleate, etc. The ricinelaidate, a geometric isomer of sodium ricinoleate which has physical properties much like the stearate and palmitate, is

TABLE 4

Effects upon Escherichi coli communis (University of Minnesota strain)

SUBSTANCE	CONCENTRATION	PLATE COUNTS		
		5 minutes	10 minutes	15 minutes
	per cent			
Germicide:				
Phenol	.83 (1–120)	0	0	0
	.71 (1–140)	200	2	0
	.62 (1–160)	4,000	250	10
	.56 (1–180)	10,000+	10,000	1,500
Saturated soaps:				
Sodium stearate	1	10,000+	10,000+	10,000+
Sodium palmitate	1	10,000+	10,000+	10,000+
Sodium myristate	1	10,000+	10,000+	10,000+
Sodium laurate	1	10,000+	10,000	8,000
Sodium laurate (pH, 8.8)	1	1,000	500	100
Unsaturated soaps:				
Sodium oleate	1	10,000+	10,000+	10,000+
Sodium linoleate	1	10,000+	10,000+	10,000+
Sodium linolenate	1	10,000+	10,000+	10,000+
Sodium clupanodonate	1	10,000+	10,000+	10,000+
Sodium alpha elaeostearate	1	10,000+	10,000+	10,000+
Sodium beta elaeostearate	1	650	400	300
Unsaturated hydroxylated soaps:				
Sodium ricinoleate	1	10,000+	10,000+	10,000+
Sodium ricinelaidate	1	10,000+	10,000+	10,000+
Sodium ricinstearolate	1	10,000+	10,000+	10,000+
Unsaturated alicyclic soaps:				
Sodium abietate (pH 9.0)	1	10,000+	10,000	8,000
Sodium chaulmoograte	1	10,000−	10,000+	10,000+
Hydroxylated salts:				
Sodium trihydroxy stearate	1	10,000+	10,000+	10,000+
Sodium sativate	1	10,000+	10,000+	10,000+
Calcium gluconate	1	10,000+	10,000+	10,000+
Bile salts:				
Sodium cholate	1	10,000+	10,000+	10,000+
Sodium dehydrocholate	1	10,000+	10,000+	10,000+
Sodium taurocholate	1	10,000+	10,000+	10,000+
Sodium glycocholate	1	10,000+	10,000+	10,000+

TABLE 4—*Concluded*

SUBSTANCE	CONCENTRATION	PLATE COUNTS		
		5 minutes	10 minutes	15 minutes
	per cent			
Aromatic carboxylic acid salts:				
Sodium salicylate	1	10,000+	10,000	8,000
Sodium diiodosalicylate	1	1	0	0
Sulfate esters:				
Sodium lauryl sulfate	1	10,000+	10,000+	10,000+
Sodium oleyl sulfate	1	10,000+	10,000+	10,000+

somewhat less effective than the ricinoleate. The ricinstearolate, containing a triple bond instead of a double bond and with physical properties very similar to the ricinoleate, is even less active than the ricinelaidate. Sodium undecylenate containing eleven carbon atoms and a double bond at the end of the hydrocarbon chain requires a fairly high concentration in order to destroy this organism.

Sodium abietate, which is an alicyclic soap and the main constitutent of rosin soap, has a destructive action on the pneumococcus in one-tenth the concentration necessary for phenol. The same is true of chaulmoograte, which has been widely heralded as a more or less specific chemotherapeutic agent in the treatment of leprosy. The hydroxylated salts, gluconate and trihydroxy stearate, seem to lack any ability to kill the pneumococci under the conditions employed. The sativate is peculiar in that the addition of a fourth hydroxyl group restores the power to destroy this organism in fairly low concentrations. The bile salts, in spite of the time honored publicity as to their ability to dissolve and kill this relatively delicate organism, are extremely inefficient in both respects as compared with other soaps. The sodium dehydrocholate, which Ziegler (1931) recommends for treating pneumonia, is in the same class as the naturally occurring bile salts in regard to its germicidal properties. Sodium salicylate requires a 5 per cent solution to kill the pneumococci in 10 minutes, while the addition of two iodine atoms to the molecule increases

its efficiency in this respect fifty times. The sulfate esters, which are now gaining wide popularity as hard water soaps, have an efficiency equal to the best of the saturated soaps in killing the organism which is the chief cause of lobar pneumonia.

The effect of the soaps upon the streptococci which cause souring of milk and which are among the most resistant to disinfection of the streptococci does not parallel the effect of the same soaps on the pneumococci. In no case, however, did it require a lower concentration to kill streptococci than to kill pneumococci, although in a few cases the values are very close. Of the saturated soaps, only the myristate and laurate exhibited killing action on *Streptococcus lactis*. The addition of one double bond as in sodium oleate gives the soap the ability to kill this organism in concentrations equal to that required by sodium laurate and myristate. The addition of a second double bond, as in linoleate, enhances this property considerably. Further addition of double bonds, however, confers no greater ability to kill streptococci. The hydroxyl group in the case of the ricinoleate enhances the bactericidal activity against the streptococcus, whereas there was a decrease against the pneumococcus. Sodium ricinelaidate shows a decrease in potency to one-tenth the value of its geometric isomer, sodium ricinoleate. The replacement of the ethylenic linkage by an acetylenic linkage, as in the ricinstearolate, caused again a marked decrease in germicidal activity. The elaeostearates, which are isomeric with the linolenate, were unable to sterilize suspensions of streptococci in concentrations as high as 5 per cent. The two alicyclic unsaturated soaps, abietate and chaulmograte, were slightly less efficacious than sodium oleate as streptococcides. Neither the bile salts nor the hydroxylated salts, with the exception of the sativate, had any killing action in the concentrations tested. Sodium salicylate did not sterilize in a concentration of 10 per cent, but the addition of two atoms of iodine enabled it to do so in a fairly high dilution. The sulfate esters were almost as effective in killing streptococci as in killing pneumococci.

The germicidal effect of each of these compounds was also tested by means of plate counts similar to those used in the tests

with *Staphylococcus aureus* and *Escherichia coli*, except that 1 per cent glucose was added to the agar for *Streptococcus lactis*, and a small amount of blood for *Diplococcus pneumoniae*. These additional tests were run in order to determine whether a zoning phenomenon, such as was observed by Eggerth (1929) in his studies, occurred with any of the soaps used in this investigation. Cade and Halvorson (1934) have also reported data which indicate such a phenomenon with certain soaps. However, in this investigation, no indication of such a phenomenon was found. The results of the tests using plates paralleled the results using growth in liquid medium as a criterion of germicidal effectiveness. These plate count experiments with the pneumococci and streptococci were run at pH values of 7.6 and 8.0, without any noticeable differences in the two hydrogen ion concentrations. The addition of sterile defibrinated blood greatly increased the amount of both the soaps and alkyl sulfate esters necessary to kill pneumococci and streptococci.

At a pH of 8.0, the effectiveness of most soaps as germicides against a resistant organism like the staphylococcus is very low. Sodium undecylenate was the most germicidal of the soaps tested. Sodium diiodosalicylate was also quite effective in killing this organism, although a 1 per cent solution did not sterilize in 15 minutes. The principal constituent of "salt water" soap, sodium laurate, also exhibited a small detrimental effect on the staphylococcus. Sodium abietate in a concentration of 1 per cent was almost completely precipitated at hydrogen ion concentrations of lower than pH 8.5. At a pH of 8.7 or greater, this soap in a concentration of 1 per cent rapidly destroyed *Staphylococcus aureus*. All the other soaps examined showed no germicidal action on this organism in a period of 15 minutes under the conditions employed in the experiment.

The three previous organisms used having belonged to a group of Gram-positive cocci, it was thought desirable to test the germicidal effectiveness of these soaps against one of the Gram-negative bacilli. Since Lutterloh and Stroud (1931) and Seely (1932) have recommended the use of sodium ricinoleate in abdominal toxemia and peritonitis, it was thought that the colon bacillus was the most

suitable of this group of microörganisms as a test organism. The effect in this case was much the same as with the staphylococci. The only compounds which killed this organism were the diiodosalicylate, undecylenate, laurate, and abietate. The action of the latter two was extremely slight.

SUMMARY AND CONCLUSIONS

Pneumococci are especially susceptible to the action of certain unsaturated soaps, such as sodium oleate, linoleate, linolenate, and clupanodonate. The other unsaturated soaps, as well as the hydroxylated and saturated soaps, are less effective in killing this organism. The bile salts and sodium salicylate require high concentrations to kill pneumococci. The sulfate ester salts are pneumococcidal in concentrations of 0.01 per cent.

Streptococcus lactis is more resistant to the action of soaps than the pneumococcus. The effectiveness of soaps in killing the latter microörganism does not parallel exactly its effectiveness against the former. The two saturated soaps, sodium myristate and laurate, the unsaturated soaps, sodium oleate, linoleate, linolenate clupanodonate, ricinoleate, and abietate, as well as the two sulfate esters, are very effective in killing this streptococcus.

Of these compounds, only sodium diiodosalicylate and undecylenate are effective germicides in a concentration of 1 per cent at a pH of 8.0, using *Escherichia coli* as the test organism.

Sodium diiodosalicylate and sodium undecylenate are also effective against *Staphylococcus aureus* at a pH of 8.0. Sodium abietate is effective above a pH of 8.5.

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RICE BRAN EXTRACTS AND THE GROWTH OF MICROÖRGANISMS

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INTRODUCTION

Working with unrefined extracts of tissues such as rice bran, Williams *et al.* (1933) reported growth increase of the Gebruder Mayer strain of *Saccharomyces cerevisiae* in excess of 15,000 fold. This increase they ascribed to a single substance which they called pantothenic acid, and which was found to be present to some extent in practically all biological tissues (Rohrman *et al.*, 1934). Based on the assumption that the stimulation was due to a single substance, we attempted to repeat with bacteria (Salle and Dunn, 1934) the work which Williams *et al.* had done with yeast. Stimulated growth and acid and gas production by bacteria thus led to two not fully substantiated conclusions; namely, that the stimulating substance for bacteria was *probably* pantothenic acid, a single entity, and secondly, that the production of acid and gas by *Escherichia coli* when grown in a medium containing rice bran extract indicated the fermentation of this single acid. It was shown, however, that the stimulation of *Escherichia coli* by unrefined rice bran extract was quite different from that by glucose. Glucose gave less growth than did the extract and a greater drop in pH; when added together with extract, growth and final pH were characteristic of the latter alone, showing that the glucose was probably not metabolized in the presence of extract.

In a test of 41 species of bacteria (Salle and Dunn, 1935) it was found that 34 out of 35 carbohydrate-fermenting organisms were stimulated by the extract, while 5 non-fermenting organisms

were not. From these results it was concluded that the stimulating substance or substances were related to the carbohydrates. Of all the hexoses, glucose is the most universally fermented by bacteria, and to our knowledge has the greatest stimulating ability of any known carbohydrate. It seems logical, therefore, to conclude that since the stimulating factor is not glucose, neither is it any other known hexose. The identity of the bacteria-stimulating agent with pantothenic acid remains a matter of conjecture.

Williams and Saunders (1934) showed that much of the growth stimulation obtained with the unrefined extracts should be ascribed not only to pantothenic acid, which they had by then obtained in a much purer form, but also to inositol, and even to vitamin B. Each of these substances exerted some stimulation in many cases alone, and a considerable stimulation in various combinations, particularly that of pantothenic acid and inositol.

A closer study of the earlier work of these writers reveals the fact that their reported stimulation of over 15,000 fold was obtained when increasing amounts of rice bran extract were added to a synthetic medium which alone gave only 11 fold growth increase over the inoculum. Since the increase was calculated upon the basis of the inoculum, the resulting values do not indicate stimulation, but only total increase of organisms during 18 hours incubation. Based upon the normal amount of growth in the synthetic medium alone, a recalculation of their data shows that the crude extract promotes growth in excess of 1,400 fold. Furthermore, growth obtained using the same basic synthetic medium, but to which was added the partially purified extract (fractional electrolysis), no longer showed outstanding stimulation, but, as calculated by us, approached a maximum of 10 fold. Since, however, it has been repeatedly demonstrated by Williams and his coworkers that such growth stimulation is accomplished by exceedingly minute traces of the partially purified substance, there can be no doubt but that the substance is a true growth catalyst. On the other hand, their data show that the addition of the partially purified extract to the synthetic medium gives only a small amount of stimulation as

compared to a like addition of the unrefined extract, the difference being due, in our opinion to the fact that crude rice bran extract renders the synthetic medium more favorable to growth by *increasing the nutritional elements*, rather than through growth catalysts.

EXPERIMENTAL

Throughout this paper the terms "growth stimulation" and "growth promotion" are used synonymously. They refer to the increase in number of organisms due to the addition to the medium of a substance or substances catalyzing the rate of multiplication. Such increase is found by dividing the amount of growth obtained in the presence of these substances by that obtained in their absence. The terms "growth" and "growth increase" are taken to mean increase in the number of organisms over the inoculum. Since in all cases the amount of growth has been determined by measuring the final volume occupied by the cells, consideration of increase in cell size should be made. However, it is our opinion, confirmed by microscopic examination, that variations of cell volume are quite regular in nutrient media. The error made in assuming constant average volume is probably less than that introduced in the process of centrifuging.

Extracts which were prepared simultaneously by the 60 per cent methyl alcohol method (as used by Williams) from old and from fresh rice bran were used to determine their respective effects as growth stimulants upon *Saccharomyces cerevisiae*. Each growth determination was made in duplicate. The growth medium included the amounts of extract, saline and yeast medium as shown in the tables. The inoculum in each case consisted of 1.0 cc. of a suspension of yeast containing 1×10^{-5} cc. of cells. The culture used for inoculation had been grown for 18 hours in the yeast medium, to which a small amount of extract had been added. The organisms were centrifuged in a Hopkins tube, resuspended to give a 0.1 per cent suspension, and then diluted to the proper concentration. The yeast medium contained the following ingredients:

Water	1000 cc.	K_2HPO_4	10.0 grams
Sucrose	50 grams	$MgSO_4$	0.5 gram
Glucose	10 grams	NaCl	1.0 gram
Levulose	10 grams	NH_4NO_3	2.0 grams
Asparagin	2 grams	$CaCl_2$	0.5 gram
		Dried beer wort	2.5 grams

This medium was adjusted to pH 6.0, filtered, and sterilized at 100°C. for 30 minutes. It was used throughout all the following experiments with yeast.

Table 1 shows the results obtained when the extract of the fresh bran was used, and table 2 the results with extract of bran which had been kept in the laboratory for over a year. Extracts from the fresh bran are shown to be superior to those from the

TABLE 1

Growth of S. cerevisiae using extract of fresh bran

Inoculum 1×10^{-5} cc. of cells per culture

EXTRACT	SALINE	YEAST MEDIUM	GROWTH CC./10 CC. MEDIUM			FOLD INCREASE (APPROXIMATE)
			1	2	Average	
cc.	cc.	cc.				
0.25	1.75	10.0	0.006	0.006	0.006	600
0.50	1.50	10.0	0.007	0.007	0.007	700
1.00	1.00	10.0	0.010	0.010	0.010	1,000
2.00	0.00	10.0	0.014	0.014	0.014	1,400

old, probably due to a smaller amount of toxic material in the former.

The growth increase was figured on the basis of the amount of the inoculum, as was done by Williams *et al.* It was inferred however, that were the inoculum decreased to one-tenth or even one-hundredth, values for growth increase might be obtained which would approach those obtained by Williams.

Accordingly, this experiment was repeated in part one week later. The extract prepared from the fresh rice bran, now 8 days old, was used. In table 3 is also reported the growth stimulating action of extract prepared by the 25 per cent ethyl alcohol method, an extraction procedure used almost exclusively in this work. In each determination as reported in the table,

10.0 cc. of the yeast medium was used. As with the other experiments, the incubation time was 18 hours.

Table 3 shows that in 18 hours the final growth is independent of the amount of the inoculum. On this basis, therefore, had the solutions reported in table 1 been inoculated with 1×10^{-7} cc. of organisms, instead of 1×10^{-5}, a maximum growth increase of 140,000 fold would have been obtained rather than 1,400 fold.

TABLE 2

Growth of S. cerevisiae using extract of one-year old bran

Inoculum 1×10^{-6} cc. of cells per culture

EXTRACT	SALINE	YEAST MEDIUM	GROWTH CC./10 CC. MEDIUM			FOLD INCREASE (APPROXIMATE)
			1	2	Average	
cc.	cc.	cc.				
0.25	1.75	10.0	0.004	0.004	0.004	400
0.50	1.50	10.0	0.005	0.005	0.005	500
1.00	1.00	10.0	0.006	0.007	0.006	600
2.00	0.00	10.0	0.007	0.007	0.007	700

TABLE 3

Growth produced with varied inocula, using two differently prepared extracts

EXTRACT	AMOUNT USED	INOCULUM	GROWTH CC./10 CC. MEDIUM			FOLD INCREASE (APPROXIMATE)
			1	2	Average	
	cc.	cc.				
60 per cent MeOH.....	2.0	1×10^{-6}	0.009	0.009	0.009	9,000
60 per cent MeOH.....	2.0	1×10^{-7}	0.007	0.011	0.009	90,000
25 per cent EtOH.....	2.0	1×10^{-6}	0.013	0.013	0.013	1,300
25 per cent EtOH.....	2.0	1×10^{-6}	0.013	0.013	0.013	13,000

Table 3 also shows that extracts which were even a week old were no longer able to give as good growth stimulation as did the freshly prepared ones. Also, the fresh extract produced by the 25 per cent ethyl alcohol process gave growth stimulation which was equivalent to that obtained with fresh extract produced by the 60 per cent methyl alcohol method. The 25 per cent ethyl alcohol extract contained slightly more solids than did the 60 per cent methyl alcohol extract. The values were

228 mgm. per cubic centimeter and 198 mgm. per cubic centimeter respectively.

Experiments with bacteria. When the extracts from the old and from the fresh rice bran were tried upon bacteria it was found that they were practically identical in stimulating ability, the latter being slightly more effective. It was concluded, therefore, that the bacterial nutrilite was not destroyed during the time that the bran had been in storage.

Experiments were performed to repeat as far as possible with bacteria the work carried out by Williams and his co-workers on yeast, and at the same time to show that the crude rice bran extract contained some, if not all, of the substances necessary for bacterial growth. Since growth in the presence of rice bran extract was always greater when the medium was buffered, Na_2HPO_4 and citric acid were added to the rice bran extracts in making up the media for these experiments, and the pH adjusted to 7.4.

This experiment was performed in five sets, each set being inoculated with amounts of bacteria which ranged by decimal dilutions from 1×10^{-3} to 1×10^{-7} cc. of organisms per culture. Resuspension and dilutions were made using the citrate phosphate buffer solution. The incubation was at 37°C. for 18 hours. From the observation of growth during the incubation period, 8 hours might just as well have been used as 18 hours. The latter length of time of incubation was adopted since it was the same as used by Williams with yeast. Ten cubic centimeters of buffer were used in each determination as reported in tables 4 and 5.

The results which are reported in tables 4 and 5 are for growths which were obtained with an inoculum at 1×10^{-6} and 1×10^{-7} cc. of organisms per culture, respectively. With three more concentrated inocula similar results were obtained.

It is quite apparent from the data shown in these tables that the crude rice bran extract is capable of supporting growth of *E. coli* when nothing else but Na_2HPO_4 and citrate are present in the medium. The extract must, therefore, contain all the necessary substances for the growth of the microörganism, with the possible exception of phosphate. (*E. coli* probably does not

utilize citric acid in the presence of fermentable carbon compounds.) In fact, this medium was more suitable for the growth of *E. coli* than was an infusion medium; the latter gave only approximately one-third as much growth per unit volume as did the buffered extract.

TABLE 4

Growth of E. coli in buffer-extract medium when the inoculum was 1 × 10^{-6} cc. of organisms

EXTRACT		SALINE	GROWTH CC./10 CC. MEDIUM			FOLD INCREASE (APPROXIMATE)
Volume	Dry weight added per cc. medium		1	2	Average	
cc.	*mgm.*	*cc.*				
0.25	4.7	1.75	0.006	0.006	0.006	6,000
0.50	9.5	1.50	0.010	0.012	0.011	11,000
1.00	19.0	1.00	0.022	0.021	0.021	21,000
2.00	38.0	0.00	0.054	0.052	0.053	53,000

TABLE 5

Growth of E. coli in buffer-extract medium when the inoculum was 1 × 10^{-7} cc. of organisms

EXTRACT		BUFFER	GROWTH CC./10 CC. MEDIUM			FOLD INCREASE (APPROXIMATE)
Volume	Dry weight added per cc. medium		1	2	Average	
cc.	*mgm.*	*cc.*				
0.25	4.7	1.75	0.005	0.006	0.005	50,000
0.50	9.5	1.50	0.011	0.011	0.011	110,000
1.00	19.0	1.00	0.022	0.023	0.022	220,000
2.00	38.0	0.00	0.055	0.054	0.054	540,000

Since *E. coli* will not grow in a medium containing citric acid and Na_2HPO_4, but no source of nitrogen, the stimulation must be figured upon the basis of the inoculum. Such calculations, although showing at first glance a phenomenal "stimulation," tell nothing as to whether the growth is due to catalysts or to nutrient substances in the rice bran extract. If, instead of extract, there had been used varying amounts of infusion medium, similar results would have been obtained, except that the amount

of 18-hour growth would have been much less. A true determination of the growth stimulation due to the extract must therefore be made only with a medium which is entirely suitable for the growth of the organism in question. In this case only can the stimulation be said to be due to a single material, such as the partially purified pantothenic acid used by Williams *et al.*

It is to be noted in table 5 that the amount of growth almost exactly paralleled the amount of extract which was added; i.e., doubling the amount of extract at least doubled the amount of growth. Such a linear increase was not observed by Williams *et al.* in their work with yeast. As they pointed out, this might have been due to the presence in the extract of substances toxic for yeast. However, except with *Rhodococcus roseus*, no toxic substances for bacteria were demonstrated by us; i.e., bacteria not stimulated were at the same time not inhibited.

Following the discovery that buffered extract medium was entirely suitable for the growth of *E. coli*, experiments were performed to determine the suitability of this medium for the growth of other bacteria. The results are given in table 6.

The rice bran extract used was prepared as follows: 800 grams of rice bran were allowed to stand for 4 days at room temperature in 3 liters of 25 per cent ethyl alcohol. The resulting mash was then filtered clear, and the filtrate evaporated on the steam bath to a syrup of approximately 25 cc. volume. This syrup was taken up in 200 cc. of 0.1 M citric acid, heated to almost 100°C. and filtered while still hot. The clear filtrate was then sterilized in the Arnold steam sterilizer at 100°C. for 30 minutes. One part of this stock solution was then added to ten parts of sterile 0.2 M Na_2HPO_4 as needed, and the pH adjusted to 7.4. Two such batches of extract were prepared for the experiment, and are designated as "A" and "B."

Although both "A" and "B" were presumably prepared by the same procedure, the amount of growth produced in the latter is in most cases superior to that produced in the former. Such variability was found to be fairly common throughout the many extractions and experiments carried out during the course of this work. Such variations have often been noted in this

TABLE 6

Comparative growth of certain species in various media

ORGANISM	EXPERIMENT 1			EXPERIMENT 2				
	Extract 2 per cent agar slant	Infusion medium	Buffered extract	Broth	Buffered extract	Extract + 0.1 per cent peptone	Extract + 0.1 per cent meat extract	Extract peptone meat extract
		cc.	cc.	cc.	cc.	cc.	cc.	cc.
E. coli	Good	0.016	0.030	0.015	0.046	0.050	0.063	0.068
E. acidi-lacti	Good	0.006	0.012	0.006	0.028	0.040	0.023	0.053
B. paracoli	Good	0.017	0.027	0.016	0.037	0.045	0.050	0.045
Aero. aerogenes	Good	0.030	0.030	0.005	0.065	0.065	0.065	0.065
Aero. cloaceae	Good	0.010	0.017	0.012	0.048	0.047	0.020	0.053
Alc. fecalis	Poor	0.018	0.008	0.030	0.024	0.032	0.030	0.031
Chr. prodigiosum	Good	0.024	0.025	0.005	0.050	0.060	0.055	0.065
Chr. violaceum	None	0.003	0.000	0.003	0.000	0.004	0.004	0.002
E. typhosa No. 3	Good	0.010	0.008	0.009	0.014	0.016	0.018	0.017
S. paratyphi (A)	Good	0.009	0.004	0.006	0.017	0.020	0.015	0.007
S. Schottmuelleri	Good	0.021	0.010	0.011	0.030	0.040	0.040	0.045
S. suipestifer	Good	0.008	0.007	0.003	0.015	0.023	0.020	0.025
S. abortivo-equi	Good	0.009	0.014	0.005	0.023	0.035	0.022	0.035
S. enteritidis	Good	0.012	0.016	0.012	0.028	0.043	0.033	0.040
S. aertrycke	Good	0.021	0.015	0.023	0.032	0.060	0.040	0.048
S. Morganii	Poor	0.010	0.006	0.006	0.005	0.027	0.012	0.024
Sh. dysenteriae	Poor	0.004	0.000	0.009	0.013	0.016	0.010	0.011
Sh. paradysenteriae	Good	0.003	0.000	0.004	0.010	0.015	0.008	0.013
Sh. paradys. Sonnei	Good	0.010	0.017	0.009	0.030	0.034	0.030	0.034
Staph. aureus	None	0.006	0.000	0.000	0.000	0.010	0.007	0.016
Staph. albus	Poor	0.003	0.008	0.003	0.017	0.017	0.014	0.014
Strep. lactis	Good	0.005	0.010	0.000	0.005	0.015	0.003	0.014
M. candidus	Poor	0.004	0.000	0.003	0.013	0.020	0.017	0.020
N. catarrhalis	Good	0.006	0.002	0.010	0.016	0.018	0.016	0.024
B. subtilis	Good	0.009	0.004	0.008	0.003	0.015	0.005	0.020
B. mesentericus	Good	0.013	0.017	0.013	0.014	0.014	0.015	0.019
B. asterosporus	Good	0.001	0.008	0.008	0.014	0.019	0.014	0.018
Pr. vulgaris	Poor	0.013	0.015	0.020	0.031	0.052	0.030	0.056
V. comma	Good	0.014	0.025	0.023	0.020	0.020	0.025	0.026
V. Metchnikovii	Good	0.011	0.017	0.008	0.038	0.049	0.036	0.052
Ps. aeruginosa	Good	0.014	0.022	0.035	0.020	0.050	0.020	0.060
Ps. fluorescens	Good	0.010	0.020	0.035	0.050	0.050	0.050	0.050
Ps. Mildenbergii	Good	0.002	0.000	0.005	0.000	0.003	0.001	0.003
Kl. pneumoniae	Good	0.013	0.020	0.007	0.022	0.030	0.020	0.050

laboratory with different batches of infusion media and with plain broth.

The infusion medium used was single strength beef infusion containing 1 per cent peptone and 0.5 per cent NaCl. The broth contained 1 per cent peptone, 0.3 per cent beef extract and 0.5 per cent NaCl. The pH of each medium was adjusted to 7.4.

Growth determinations were made by centrifuging 10.0 cc. of each culture in a Hopkins tube for 45 minutes. Two centrifuges which had approximately the same rotational velocities were employed. Cultures were incubated at 37°C. for 24 hours and following incubation were kept in the ice box until growth was determined, a procedure necessitated by the considerable number of determinations which had to be made.

Experiments 1 and 2 (table 6) were performed at different times and with different media. The results of 1 are therefore not comparable with those of 2, but since in both experiments 0.2 cc. of an 18-hour plain broth culture of each organism was used for inoculation, comparisons within each group are possible.

In experiment 1 growth in two liquid media (infusion and buffered extract A), and growth on 2 per cent agar buffered extract medium are reported. Most organisms were found to grow better in the rice bran extract medium than in the infusion. Several did not grow at all in the extract medium, but did grow upon the extract agar slants. Only two organisms, *Chromobacterium violaceum* and *Staphylococcus aureus* gave no growth upon the solid medium.

In experiment 2, five liquid media were used; namely, plain broth, buffered extract B, buffered extract B plus 0.1 per cent peptone, plus 0.1 per cent Liebig's meat extract, and plus both 0.1 per cent peptone and 0.1 per cent meat extract. Quantitative results show that of these five media, the buffered extract plus 0.1 per cent peptone was able to promote growth at least to the same extent as the buffered extract, and in the majority of cases to a much greater degree. On the other hand, the addition of 0.1 per cent meat extract or the addition of both peptone and meat extract in almost half the cases did not increase the amount of growth over that obtained with the buffered extract medium alone. It is therefore concluded that the most generally satisfactory medium, from the standpoint of cost as well as amount

of growth, is that containing buffered extract and 0.1 per cent peptone. This medium is probably able to give more prolific growth of all carbohydrate-fermenting organisms than is any other medium in general use in the bacteriological laboratory at the present time.

In addition to those organisms listed in table 6, the following organisms gave good growth upon the extract agar slants, but no growth in 24 hours in the liquid rice bran extract medium: *Mycobacterium smegmatis*, *Staphylococcus luteus*, *Sarcina lutea*, *Micrococcus lysodeikticus*, *Staphylococcus citreus*, *Brucella melitensis*, and *Brucella abortus*. All of these organisms required more than 48 hours for growth to develop upon the solid medium. *Rhodococcus roseus* showed no growth whatsoever in any extract media, confirming previous results with regard to this organism. *Brucella abortus*, *Brucella melitensis*, *Staphylococcus luteus*, and *Micrococcus lysodeikticus* grew sparsely in the liquid rice bran extract media when incubation was continued for 72 hours or more.

In view of the results which tended to show that only carbohydrate-fermenting organisms were stimulated, it seemed important to determine more extensively whether, out of a larger group of species of bacteria, all those having fermentative properties would produce acid and gas, or at least acid in a medium containing rice bran extract. Accordingly fermentation tubes containing extract broth, Andrade's indicator, and 5 per cent rice bran extract were inoculated with the several organisms. In 24 hours incubation 14 produced acid and gas, 19 produced acid alone, 6 produced slight amounts of acid, and 10 showed no fermentation. The total number of organisms tested throughout these many experiments was now 65. Of this group, 9 were non-fermenters of sugars. These latter together with *Shigella dysenteriae* composed the group which showed no acid production in the fermentation tubes.

Analysis of the results obtained with the rice bran medium indicates that the growth of most organisms is supported by it, and that the growth of those organisms which have fermentative powers is greatly enhanced. For those workers whose experi-

ments require large quantities of such organisms as are stimulated, or who desire an increased rate of growth of such bacteria, the 0.1 per cent peptone rice bran extract medium is recommended.

CONCLUSION

1. Rice bran contains a stimulating substance or substances for carbohydrate-fermenting bacteria and for yeast.

2. Pantothenic acid may be one of the substances responsible for the growth stimulation of bacteria.

3. The stimulating agent may be related to the carbohydrates, but it is not glucose nor any other hexose which stimulates *Escherichia coli* in a manner similar to glucose.

4. Old extracts showed less stimulating ability both for bacteria and for yeast while fresh extracts prepared from fresh and from old bran were found to be equally effective in stimulating bacteria, but only extracts prepared from the former gave good yeast stimulation.

5. Extracts prepared by the 60 per cent methyl alcohol extraction method, and by the 25 per cent ethyl alcohol method are equivalent in stimulating ability.

6. These rice bran extracts contain, with the possible exception of phosphate, all substances necessary for the growth of *Escherichia coli* and many other organisms.

7. Better growth of most organisms was obtained with buffered extract medium than was obtained using ordinary bacteriological infusion medium.

8. The use of 0.1 per cent peptone rice bran extract medium is recommended for obtaining greater growth of carbohydrate-fermenting bacteria, or for increasing the rate of growth of such types.

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THE STATUS OF THE GENERIC TERM BACTERIUM EHRENBERG 1828*

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The status of the generic term *Bacterium* needs to be clarified by international action, as the status of *Bacillus* has been clarified by the action taken in regard to the proposals drawn up by Buchanan and Conn and reported upon by Breed and St. John-Brooks (1935). The history of *Bacterium* has been well outlined by Buchanan in his Systematic Bacteriology (1925). In this he quotes with approval the suggestion made by Breed, Conn and Baker (1918) that a temporary group be recognized to include species of non-spore-forming rods whose relationship to other species is obscure.

As the type species concept has now been accepted as a method of defining generic terms (Art. 18, International Rules of Botanical Nomenclature adopted by the Fifth International Botanical Congress, 1930) suggestions that *Bacterium* be used for (1) non-spore-forming rods (Zopf[1]), (2) non-motile rods (Migula), (3) fluorescent pseudomonads (Smith, Vuillemin), (4) the colon-typhoid group (Orla-Jensen, Winslow *et al.*), (5) gram-negative, non-spore-forming rods (Lehmann and Neumann, 1927) are invalid on the ground that the type species *Bacterium triloculare* Ehrenberg, 1828 (monotypy) is unrecognizable. Because this species is unrecognizable, subsequent authors have felt free to redefine the genus. As none of these new definitions has won universal approval, it is necessary to state how the term is being used each time it is used or confusion results.

This situation pointedly illustrates the need for the rule that

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[1] See Buchanan, R. E. (1925) for references not given at the end of this paper.

generic terms not associated with a recognizable type species are invalid and has already led many authors to discard the term (Baumgarten, Sternberg, Fischer, Matzuschita, Schneider, Castellani and Chalmers, Bergey *et al.*, Pribram, 1929); and has caused others (Lehmann and Neumann, 1927, Jordan, 1935) to use it as a more or less temporary name.

Many difficulties in classification disappear where *Bacterium* is retained as a temporary genus, without valid status, to include species of non-spore-forming rods that cannot readily be placed in well recognized and well defined genera. We therefore wish to develop the suggestion made in our 1918 paper into a definite recommendation that *Bacterium* be accepted as a temporary generic term with an admittedly unrecognizable type species *Bacterium triloculare* Ehrenberg to include those species of non-spore-forming, rod-shaped, motile or non-motile bacteria whose relationships to other bacteria are not clear. When relationships to other similar species have been developed by investigation, then this generic term would naturally be dropped.

This would permit, for example, the retention of such a species as *Bacterium radiobacter* in the genus *Bacterium* until its systematic position is better understood without the necessity of placing it in *Achromobacter*; and many similar changes in names could be avoided. It would also give justification to those who wish to be conservative and retain such names as *Bacterium coli*, *Bacterium aerogenes*, *Bacterium violaceum*, *Bacterium amylovorum*, *Bacterium zopfii*, *Bacterium monocytogenes*, *Bacterium pneumoniae*, etc. Those who accept *Bacterium* as a temporary genus could continue the use of binomials such as those quoted above in full conformity with accepted and acceptable usage.

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A NOTE ON CONDITIONS AFFECTING THE PRODUCTION OF TOXIN AND PORPHYRINS BY THE DIPHTHERIA BACILLUS[1]

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This paper records observations made in a further study of the relationship of "porphyrins" and toxin in diphtheria cultures. As reported in a recent publication (Wadsworth, Crowe and Smith, 1935), these products of growth or metabolism appear to be present or absent under identical conditions and yet to be quite distinct and separable. In these early experiments, however, the cultures examined were, for the most part, cultures of toxigenic or nontoxigenic strains grown under the conditions routinely employed for toxin production—that is, incubation at from 32° to 35°C. for from seven to ten days under ordinary atmospheric conditions. The work has since been extended to include cultures grown for different periods of time and under varied conditions.

Filtrates of cultures of a toxigenic strain of the diphtheria bacillus grown in infusion-free peptone medium for periods of time varying from eighteen hours to three weeks have been examined for the presence of toxin and of porphyrins. Toxicity determinations were made by both the intracutaneous method and the flocculation reaction, and ether extracts prepared according to the method of Hans Fischer—usually from 500 cc. of filtrate—were examined for absorption in the visible region of the spectrum. Selective absorption was observed for the extracts from all of the filtrates in which toxin was found. Demonstrable amounts of toxin and porphyrins usually appeared in the cultures

[1] Presented before the Society of American Bacteriologists, New York, December 28, 1935.

within the first twenty-four to forty-eight hours of incubation. The amount of each increased up to about the seventh day. No appreciable decrease of either was observed in cultures incubated for as long as three weeks.

Cultures incubated in sealed containers in which varying amounts of carbon dioxide or oxygen were present have also been examined. The approximate amounts of these gases present at the beginning and end of the period of incubation were determined by gas analysis.

When cultures of either toxigenic or nontoxigenic strains were incubated in an atmosphere which contained less than 1 per cent each of oxygen and carbon dioxide, there was definite growth but little or no toxin was produced. A culture of a toxigenic strain incubated in an atmosphere containing between 1 and 5 per cent of each gas was about half as toxic as the control aerobic culture. A culture incubated in an atmosphere to which approximately 10 per cent carbon dioxide was added had practically the same toxicity for guinea pigs as the control aerobic culture, but its flocculation value was appreciably less.

Ether extracts from 500 cc. of each filtrate of the cultures grown under these different atmospheric conditions were examined qualitatively in the visible portion of the spectrum for the presence of absorption bands. Definite absorption bands were observed for the extract of every filtrate in which there was an appreciable amount of toxin and all of the bands occupied positions at or near those in the absorption spectra of some of the porphyrins. The positions of the absorption maxima in the spectra of the ether extracts from the filtrates of cultures grown under conditions other than ordinary atmospheric conditions, however, were not the same; they also differed from spectra for the extracts from filtrates of either high or low potency from cultures which were grown under the usual atmospheric conditions. No definite bands were observed for the extracts from the nontoxic filtrates of the cultures grown in an atmosphere with less than 1 per cent each of oxygen and carbon dioxide. All extracts which showed bands fluoresced with a red light, while the extracts from the nontoxic filtrates fluoresced green.

In the earlier paper it was also reported that it was possible by treatment with charcoal to remove practically all of the porphyrins from a toxin without significantly changing its potency. Experiments were therefore made to determine the effect of the presence of charcoal in the medium on growth and toxin production. It was found that potent toxins may be produced in a medium which contains 1.5 per cent of an ash-free charcoal preparation and that these toxins are practically free from porphyrins. We have not succeeded, however, either in recovering porphyrins from the charcoal or in demonstrating their presence on it.

The effect of the porphyrins, extracted by ether from a highly potent diphtheria toxin, on the growth and toxigenic activities of toxigenic and nontoxigenic strains has also been tested. The residue obtained on evaporating the ether extract in a vacuum desiccator was dissolved in a sodium-acetate solution, sterilized by filtration, and added to the medium at the time of inoculation. The amount of porphyrins added to each flask of medium was equivalent to that extracted from an equal volume of the toxin. This amount did not appear to affect the growth or the toxigenic activities of either the toxigenic or nontoxigenic strains tested. An ether extract of an uninoculated control medium to which the porphyrins had been added and which had been incubated for ten days, contained an appreciable amount of pigment. In general, too, all of the extracts of cultures grown in the medium with porphyrins appeared to contain a somewhat larger amount of pigment than the extracts of the corresponding control cultures grown in medium without porphyrins.

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THE INCIDENCE AND SIGNIFICANCE OF THE PRESENCE OF BORRELIA VINCENTI AND OTHER SPIROCHAETACEAE ON BEVERAGE GLASSES[1]

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Dentists and Public Health Workers have lately reported a marked increase in the number of cases of Vincent's infection, popularly called Trench Mouth. Dr. Davis (1935) of the Michigan State Board of Health made the following statement at the recent annual meeting of the Michigan Public Health Association, "There is a rather widespread opinion among Health Officers and some physicians and dentists that Vincent's infection is on the increase. Perhaps we are going through the process of its becoming endemic." Letters written to the State Boards of Health throughout the country regarding the incidence of the disease have brought the information that where some attempt is made to enforce reporting of cases, this statement is emphatically verified. For example, in the State of Washington seven cases were reported in 1931 and 343 in 1934; in Illinois 95 cases were reported in 1931 and 758 or a 700 per cent increase in 1934; 880 cases were reported in up-state New York in 1931 and 1733 cases in 1934 or a 100 per cent increase. Naturally these figures are subject to some question in as much as the possibility of increased recognition of the disease, better coöperation in reporting etc., may enter into their significance.

These figures may, however, indicate a new active source of the disease which is spreading the infection, as well as more frequent transmission from individual to individual. Our present knowledge of this disease is predicated on the basis that it is

[1] Presented before the Society of American Bacteriologists, New York City, N. Y., December, 1935.

spread directly by contact with the mouth of the infected individual, or with salivary moisture, or indirectly by the use of insufficiently cleansed eating utensils, glassware etc.

In searching for a possible explanation of these facts which would account for a widespread source of contagion, attention was called to the report of Drs. Mallman and Devereaux (1935) regarding the bacterial counts which they found on beverage glasses. Working with Drs. Mallman and Chandler the author followed up this work with an examination of beverage glasses with regard to the possible presence of various types of spirochetes.

Mallman and Devereaux found a deplorable lack of proper methods of cleaning and sterilizing beverage glasses in the many insufficiently-regulated taverns, road-side stands, and saloons which have sprung up since the repeal of the 18th Amendment and reported astonishingly high bacterial counts on the rims of supposedly clean glasses racked upon the counter or just out of the rinse water and ready to be refilled for customer use.

The first step in this examination was to devise methods of obtaining satisfactory samples from the glasses which could be subsequently stained and examined microscopically. Small cotton swabs were prepared on long wooden applicators and placed in test tubes, which were sealed with a cotton plug and sterilized by autoclaving. The glasses were tested by wiping the rims inside and out with this swab which was then dropped in sterile test tubes containing 1 cc. of a 0.1 per cent solution of suspensoid colloidal iodine (Chandler). The swab was washed in this solution and then squeezed dry against the walls of the tube.

This instantly killed and fixed the bacteria preventing their lysis or growth during the time elapsing between sampling and laboratory examination. Then, 10 cc. of distilled water were added to this solution and the tubes centrifuged at 2500 r.p.m. for 25 minutes, after which the supernatant fluid was poured off and the residue examined both by dark field and by staining.

Samples from two hundred glasses obtained in eight representative beverage-dispensing establishments in two different cities were examined in this manner with the following results:

Found on rims of glasses:

	Number	Per cent
Spirochaetaceae	38	19
Borrelia Vincenti	24	12
Treponema macrodentium	10	5
Treponema microdentium	4	2

In all smears many types of cocci and bacilli were found. *Fusiformis dentium*, the organism usually found symbiotically with the *Spirochaetaceae* in Vincent's infection was demonstrated in only one instance. In one instance an amoeba was demonstrated, presumably the *Endamoeba gingivalis*.

The findings in establishment number (1) are characteristic of a representative type of beverage dispensory. Here, 62 glasses which had just returned from the patrons were examined before they were rinsed. In eight instances *Borrelia Vincenti* was found, in two *Treponema macrodentium*, and *Treponema microdentium* once. This establishment was making every effort to clean the glassware effectively and was using a large electric dish washer and hot water. When sufficient glasses accumulated they were placed in this washer and the machine turned on. After a period of five minutes, a small amount of a chlorine preparation was added and the washing continued five minutes longer. Thirty glasses examined which had just been removed from this washer showed no spirochetes or other bacteria.

In representative establishment number (2) 22 glasses were examined as they returned from the customer. On three, or 14 per cent of these, *Borrelia Vincenti* was found. Four of 20, or 20 per cent of the glasses racked behind the bar and ready to be refilled for customer use, were found to be infected with this spirochete. This finding agrees with the report of Mallman and Devereaux that is some instances empty glasses returning from customers are cleaner than those which have been presumably washed and racked for refilling.

In 16 instances rinse waters were examined and although many types of bacteria have been found, *Spirochaetaceae* have not yet been demonstrated. We have no evidence that the microörganisms in this particular case were viable when recovered from the glasses since no attempt was made to cultivate them.

DISCUSSION

This report suggests the necessity for more adequate regulations in glassware sterilization in taverns, saloons, etc., and demonstrates the need for sterilizing agents which are effective in all instances or for some different method of dispensing alcoholic beverages. It also suggests the possible source of many of the cases of Vincent's infection now developing.

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EXPERIMENTAL POLIOMYELITIS INDUCED BY INTRACUTANEOUS INOCULATION[1]

A STRAIN OF THE VIRUS APPARENTLY PECULIARLY INFECTIVE WHEN INJECTED BY THIS ROUTE

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The inoculation of the virus of poliomyelitis by the intracerebral route has generally been acknowledged as the most constantly effective method of inducing experimental poliomyelitis in the monkey. The nasal passages represent another route effective with some strains of the virus. Earlier procedures also included the intraperitoneal and the intraneural routes; in the latter case the virus was usually inoculated into the sheath of the sciatic nerve. Some difficulty seems to have been encountered in inducing the disease by "extraneural" routes (International Committee, 1932, pp. 80–87; 130–144). It may suffice to say that according to most observers, feeding the virus (with the intestines uninjured) is seldom effective; its inoculation by the intravenous route is also nearly always ineffectual although recently Lennette and Hudson (1935) have described infection following repeated intravenous inoculation. On the other hand the experimental disease has often been produced when the virus has been given sub- or intracutaneously. With regard to the intracutaneous route, one observer (M. Brodie) has been quoted (International Committee, 1932, p. 83) as saying that: . . . "when a potent virus is used (such as the M.V. strain), infection may be obtained consistently by this route with an average incubation period of 8 days. . . . The total amount of virus to be used is injected in one bleb; 15,000

[1] Supported by a grant from the President's Birthday Ball Commission for Infantile Paralysis Research.

to 20,000 times as much infective cord material was required as for a successful intracerebral inoculation." It is unknown, whether or not Brodie's observation on the consistency with which massive doses of highly virulent "passage" strains will infect intracutaneously, has been repeated. However, the results of many active immunization experiments on monkeys with either live virus, or virus treated by chemical or physical agents, point to the fact that with the different doses employed in these experiments only a small percentage of the animals thus injected succumbed to the experimental disease as a result of this injection (International Committee, 1932; Brodie, 1935a and b; Kolmer, 1934). On the other hand Flexner (1935) has stated that: "When human virus is injected successively into the skin of *macacus* monkeys, it produces active immunity in the greater number, but paralysis in a proportion of the inoculated animals, just as the passage strains do."

Olitsky and Cox (1936) observed experimental poliomyelitis in 2 animals following the subcutaneous inoculation of virus, after it had been treated with tannin.

That some strains of poliomyelitis virus will differ in their ability to produce the experimental disease when injected intracutaneously in relatively small doses is the subject of this report. The observation was made during the course of a series of active immunization experiments on monkeys with six different strains of poliomyelitis virus. The technique consisted in the preparation of 10-per-cent cord suspensions representing each of the six different strains. One or more samples from each strain were exposed to 0.1 per cent formalin (one part 40 per cent formaldehyde in 1000 parts of saline solution) for 24 hours at ice-box temperature. Intracutaneous inoculations were made with two 2 cc. doses of the 10-per-cent virus suspensions, the second one being given two weeks after the first. The material was injected into the shaved skin of the abdomen in a series of 10 piqures. Daily temperature readings were taken over a period of six weeks on all animals thus inoculated. The effect of these inoculations in 20 monkeys is listed in table 1. In the majority of instances no symptoms followed the injections. With the F. strain (a virulent strain in its eleventh passage) 1 out of 4 inoculated animals

became febrile on the 9th day, and was completely paralyzed 2 days later. This animal had been inoculated with the virus untreated with formalin. Of the 5 animals inoculated intracutaneously with the Wfd. strain (a much less virulent strain, as far as its intracerebral infectiveness is concerned) 4 developed symptoms in from 9 to 17 days after the first inoculation. These symptoms were characterized by fever, tremor and ataxia. Two

TABLE 1

Incidence of experimental poliomyelitis in monkeys inoculated intracutaneously with six strains of the virus

STRAINS			ANIMALS INOCULATED WITH:		ANIMALS WHICH DEVELOPED SYMPTOMS FOLLOWING:		TOTAL INCIDENCE OF ANIMALS DEVELOPING EXPERIMENTAL POLIOMYELITIS
Name	Origin	Passages in monkeys	Formalinized virus	Live virus	Formalinized virus	Live virus	
Wfd.	Spinal cord. California, 1934	3	2	2	2	1	4/5
		5		1		1	
Mc.*	Nasopharynx. California, 1934	3	2	1	0	0	0/3
W†	Nasopharynx. New Haven, Ct., 1931	9	2	1	0	0	0/3
F.†	Spinal cord. New York, 1931	11	2	2	0	1	1/4
Park† (mixed strain)	Spinal cord	Many	2	1	0	0	0/3
Aycock†	Spinal cord	Many	1	1	0	0	0/2

* The isolation of this strain has been described recently by J. R. Paul, J. D. Trask and L. T. Webster: Isolation of poliomyelitis virus from the nasopharynx, Jour. Exp. Med., **62**, 245, August, 1935.

† The properties of these strains have been described by J. R. Paul and J. D. Trask: Neutralization test in poliomyelitis, Jour. Exp. Med., **61**, 447, April, 1935.

of the 4 monkeys also developed paralyses. Extensive paralyses did not occur and none of the animals died as a result of this illness. Only 1 of these 4 monkeys was sacrificed. Histological examination revealed extensive lesions characteristic of poliomyelitis, in the medulla, lumbar and cervical cord. The disease exhibited by these 4 monkeys did not differ materially from that produced by intracerebral inoculation of this same strain of virus in its earlier passages, except that after intracerebral inoculation

paralyses were more extensive and the incubation period was shorter.

The Wfd. strain is a so-called human strain. It was used on its third and fifth passage. It had been isolated in California in June, 1934, from the spinal cord and medulla of a fatal human case of poliomyelitis.[2] The first three passages to which it had been subjected were made in June and October, 1934; the fourth and fifth passages were done in July, 1935. The intracutaneous inoculations with the third passage were made in the period of February to June, inclusive, 1935; the one inoculation with the fifth passage was done in September, 1935. It will be noted that this Wfd. strain had been subjected to comparatively few monkey passages, but whether its enhanced infectivity by the cutaneous route is due to some specific property, or to its proximity to the human host is unknown. It is also of some interest to note that the formalinized virus (Wfd.) was infective, or in other words, the procedure of formalinization to which it had been subjected was insufficient to kill the virus completely. Its viability after exposure to formalin was not tested by intracerebral inoculation.

We do not believe that the cutaneous infectivity of this strain of low virulence is to be compared with that mentioned by Brodie and obtained by massive dosage. The cutaneous infectivity of the Wfd. strain seems to be a unique property which does not have to do with high virulence as tested by the intracerebral method. Whether this property will be retained in subsequent passages will be the subject of further study.

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[2] We are indebted to the Superintendent and to the Medical and Pathological Staffs of the Los Angeles County General Hospital for the material from which this strain was obtained.

A SUGGESTED STANDARD METHOD FOR THE INVESTIGATION OF ELECTROPHORESIS

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Although the number of papers on electrophoresis is rapidly increasing, the errors in technique to be found in many of them would seem to make a discussion of the necessary safeguards in using such methods desirable. Numerous techniques are available in the literature but the one described in this paper has been used with such uniform results by several investigators, over a period of ten years, that it seems advisable to advance it as a possible standard procedure and to summarize in one place the method in some detail. Many of the developments involved have not been previously described.

THE ELECTROPHORESIS CELL

Choice of cell. For quantitative studies, microelectrophoresis cells are to be preferred to the U-tube type in regard to simplicity of theory and ease of operation (Abramson, 1932, 1934a). Both flat and cylindrical microelectrophoresis instruments are available but, of the two, the flat cell is better suited for routine measurements. The theory of the cylindrical cell is slightly simpler but to use it properly an optical bench is required. In addition, errors in leveling are hard to eliminate because the "stationary" level is a horizontal cylinder in form. This means that particles in the same microscope field are at different levels unless the focus is directed exactly on the high point of the cylinder. The flat cell is much easier to handle.

The author knows of three types of flat cells in general use which can be made to yield good results in proper hands. These

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are, the Abramson cell (Abramson, 1929a, 1934a, Abramson and Grossman, 1931), the modified Northrop-Kunitz cell described by Mudd, Lucké, McCutcheon, and Strumia (1928) (see also Moyer, 1934a), and the modified Buzágh cell described by Bull (1935). Of these three, the Abramson instrument is by far the best, in the opinion of the writer. The Buzágh cell is a cemented cell and has to be taken apart to be cleaned. Furthermore, if both top and bottom are not of identical glass, the depth parabola may not be symmetrical unless proteins are being investigated. High magnification is difficult to use with this cell. The modified

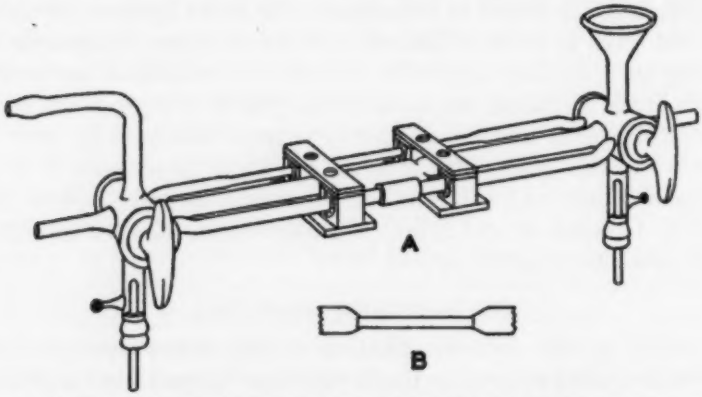

FIG. 1. *A*, THE ABRAMSON MODIFICATION OF THE NORTHROP-KUNITZ ELECTROPHORESIS CELL; *B*, SIDE VIEW OF CENTER CHAMBER

Northrop-Kunitz cell has a center chamber identical in construction with the Abramson instrument but its rubber connections make it difficult to clean and much more troublesome to use. It is particularly affected by vibrations in the neighborhood. The Abramson cell is of one piece of glass, as shown in figure 1. This permits rapid cleaning with NaOH and dilute acid or cleaning fluid. Since the instrument rests directly on the stage of the microscope, it can be inverted, a procedure of advantage in investigating large particles (Abramson, 1929b). The method of making the rectangular center chamber has been described by Mudd, Lucké, McCutcheon, and Strumia (1928) as follows:

The top and bottom of the cell are formed by thin microscopic slides; these are held 0.6 to 0.8 mm. apart by narrow glass strips cut from similar slides. The margins of the upper and lower slides and the glass strips between them are pressed together by a specially constructed metal clamp and are fused by a hot, minute, pointed flame. The cell is then completed by fusing glass tubing onto its two ends. . . .

The construction of the side arms permits the introduction of plaster of Paris plugs to prevent precipitation of proteins and sols by diffusion of the $CuSO_4$ from the electrode compartments into the center. The instrument can be adapted to use with the slit ultramicroscope or paraboloid condenser if desired. It is particularly well suited for the investigation of proteins, whereas difficulties may be encountered with the other two cells. Abramson and Grossman (1931) present dimensions of typical cells.[2]

Construction of frame. A frame for the instrument has never been described. It has been found easy to make such a frame from pieces of brass, as shown in figure 2. The uprights were soldered on the base plates so that the struts (of glass rod) ran through the deep notches filed in them. The base plates were placed just far enough apart to fit the mechanical stage of the microscope and then slightly warmed on a hot plate. While warm, the instrument was placed down into the notches and leveled. The notches were filled with melted cement (picein, de Khotinsky, etc.) and the top plates, which were provided with soldered risers to clear the center arms, were then bolted on while the cement was warm. Flat-head machine screws with a sleeve nut (of the type used for anchoring bolts in plaster) were put in, flush with the top and bottom as shown (fig. 2, *b*). If desired, leveling screws may be inserted at convenient places on the frame, although if care is taken this is not necessary. Two brass clips screwed on the mechanical stage of the microscope hold the instrument in place.

Preparation of electrodes and calibration. Cu-$CuSO_4$ electrodes are most satisfactory. Zn-$ZnSO_4$ electrodes are liable to form some gas. The plaster of Paris plugs are made by placing a little

[2] The Abramson cell may be obtained from Mr. J. D. Graham, Haddonfield, New Jersey.

dry plaster into the electrode vessels and adding sufficient water to make a plug approximately $\frac{3}{4}$ inch thick. After these have set, the apparatus is filled with saturated Na_2SO_4 and 10 milliamperes are sent through the system until the current no longer increases (about two hours). Saturated $CuSO_4$ and the copper electrodes are added and the outlets sealed with picein as described by Abramson (1929a). The rubber caps for vaccine bottles have been found to be better than rubber stoppers in the electrodes because creepage is eliminated. The electrical circuit is led to two binding posts on a block near the microscope. From there,

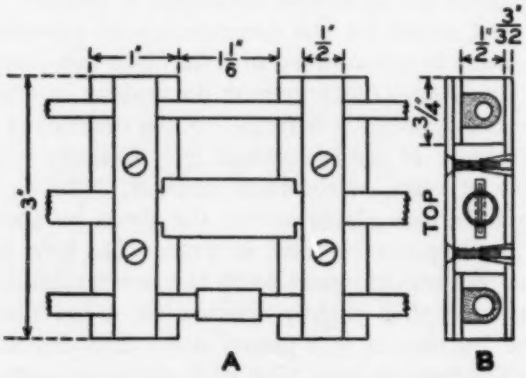

FIG. 2. CONSTRUCTION OF THE BRASS FRAME FOR THE INSTRUMENT
A, top view, showing frame in place. *B*, section through side view. Cemented area stippled.

magnet wire and small battery clips are connected, when desired, to the heavy bus-bar electrodes.

A water-cooled lamp, 25× ocular, and a Zeiss 40× water-immersion objective with a 1.6 mm. working distance provide the best optical system. The microscope must be fitted with a graduated fine adjustment. This must be calibrated, either with a cathetometer or by a standard thickness slide. A standard thickness slide may be made by cementing a flat, thin coverslip on a slide with hot balsam, measuring the thickness of both the double and single layers of glass with a micrometer caliper, and subtracting. This value of the thickness of the coverslip can be compared with the value on the fine adjustment by focussing first on the

top of the coverslip and then moving to the slide and focussing down on it with the fine adjustment. The fine adjustment is left set at this point and the coverslip is again measured by focussing on its top with the coarse adjustment and, after moving, bringing the slide into focus by turning the fine adjustment. This distance from the end of the drum represents the apparent thickness of twice the coverslip. By plotting the true values of these successive thicknesses against the apparent values (as measured on the drum), the correction factor is obtained and the linearity of the fine adjustment checked. The curve will probably be nearly linear over the small portion of the range used. In operation, it has been found practical to use only the top of the range and to start all depth measurements from the ceiling of the cell. The width of the cell may be measured by focussing on one edge and then after moving to the other edge by the mechanical stage, the difference is read on the vernier. The depth is determined with either a 21× dry objective and an empty cell or with the 40× water immersion and the cell full of water. The cross section area is obtained by multiplying the inside width at the center by the average depth measured at different points across the width of the cell. If calibration electrodes are present in the cell, this area may be measured electrically (Moyer, 1934a, 1934c).

DETERMINATION OF FIELD STRENGTH

Mobilities may be expressed as μ/sec./volt/cm. or, if the viscosity, η, or dielectric constant, D, are not the same as in distilled water, it may be better to convert mobilities to millivolts by the Helmholtz equation,

$$\zeta = \frac{4\pi\eta}{D} V, \tag{1}$$

all units being c.g.s.-e.s.u. In this connection see Abramson (1934a) and Abramson and Moyer (1936). In any case, the field strength, X (in volts per centimeter), in the center chamber must be measured. This is done by applying Ohm's law,

$$X = \frac{IR}{q}, \tag{2}$$

where I = current; R = specific resistance of the suspension; q = cross section area of the rectangular chamber in the instrument. Abramson (1929a), Abramson and Grossman (1931), and Moyer (1934c) have tested the applicability of this expression to cells of this character. It is important to stress the measurement of current. Abramson and Grossman (1931) have shown that a change in field strength of over 54 per cent may take place in

TABLE 1

Mobilities of bare quartz particles in 1×10^{-4} M KCl, illustrating the need for correcting for changes in the field strength

The time values represent the time required for a single particle to travel a fixed distance. The last column has been corrected to a field strength corresponding to 15.1 microamperes.

OBSERVED TIME	CURRENT	APPLIED VOLTAGE	CORRECTED TIME
seconds	*microamperes*	*volts*	*seconds*
7.0	15.1	20	7.0
7.2	16.5	20	7.9
7.4	16.6	20	8.2
7.4	16.8	20	8.2
6.4	17.8	20	7.6
6.4	18.1	20	7.7
6.8	18.3	20	8.2
6.0	18.5	20	7.4
6.2	18.5	20	7.6
6.0	18.7	20	7.4
6.3	18.7	20	7.8
5.7	19.4	20	7.3
6.0	19.4	20	7.7
5.6	19.6	20	7.3
5.6	19.8	20	7.4
5.9	20.0	20	7.8
			Mean 7.65

experiments carried on in a wide range of ionic strengths. During this change, the applied voltage was completely constant. Furthermore, because of slight changes at the electrodes, the voltage drop in the chamber may change 40 to 50 per cent during the course of a single set of measurements. This change is likewise not reflected by corresponding changes in the applied voltage. It is therefore not sufficient to measure the applied voltage alone

and call the drop in the cell a constant fraction of the total drop, although many investigators have done so.

To demonstrate this, some of the finely ground, bare quartz used by Bull (1935) was suspended in 1×10^{-4} M KCl. The current, voltage, and time required for a particle to travel a fixed distance were measured simultaneously. As shown in table 1, after time values were corrected for changes in field strength they

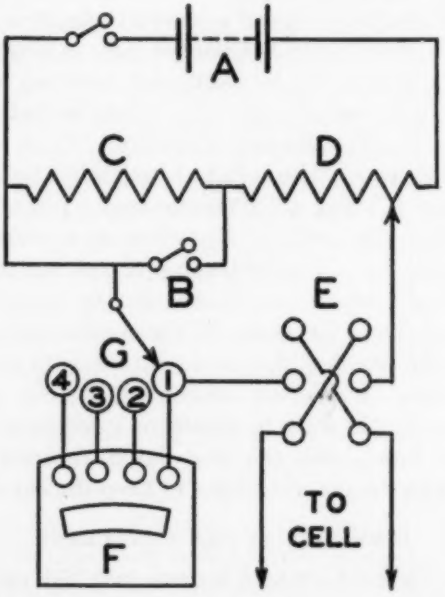

FIG. 3. WIRING DIAGRAM FOR CONTROLLING THE FIELD STRENGTH
These parts are conveniently mounted on a panel

became constant. At high dilutions, it has been frequently assumed that the high resistance of the solution makes it possible to ignore the resistance of the electrodes and call the drop in the cell a constant ratio of the applied voltage. The low salt concentration in the experiment was chosen to show how much the field strength varies, even under these conditions. As reported by Abramson (1934a), there was no orientation of particles or

observable influence of size and shape on mobility. Greater constancy would, however, be attained in less dilute solutions.

The measurement of specific resistance is done with a simple Wheatstone bridge. The "slide wire" may be made of two equal 1000-ohm fixed resistances costing less than a dollar. The conductivity cell may be made from a pipette.

It is convenient to keep the current constant while measuring and for this purpose an inexpensive resistance unit has been constructed. The wiring diagram is shown in figure 3. The source of potential is three radio B batteries (*A*), in series, giving 135 volts (D.C. house current is usually not constant enough). By closing the toggle switch (*B*), the available voltage range is changed from 67–135 volts to 0–135 volts. There is less current drain when *B* is open. The voltage is controlled by a 10,000-ohm fixed resistance (*C*) and a 10,000-ohm radio potentiometer (*D*). The D.P.D.T. spring switch (*E*) (wired as a reversing switch) reverses the polarity of the electrophoresis cell but does not affect the ammeter (*F*) which can be left in the circuit. A circuit selector (*G*) changes the range of the milliammeter. At *G*, *1*, the meter is disconnected; *2*, *3*, and *4* successively introduce more sensitive ranges. A Weston milliammeter (No. 280), of the ranges 0–1.5, 15, 150 ma., is satisfactory for most work. The operator's left hand holds the stop watch, reverses the current, and manipulates the potentiometer to keep the current constant.

OPERATION OF THE INSTRUMENT

In practice, the field strength is made just high enough for convenient timing but not high enough to produce turbulence. The particle under observation is allowed to move between two lines in the eyepiece micrometer (preferably of the cross-hatch variety) and then reversed and brought back. The objective is always focussed with the cell in the same position. This is done by always bringing the mechanical stage to the same place.

No difficulties due to precipitation of the sol by diffusion of electrolytes from the electrodes have ever been noted. Contrary to many other cells, the instrument may be operated continuously for over an hour without change in the results (the curves reported by Moyer and Abramson (1936) were obtained in this way).

Distilled water should be used with caution as a suspending medium. Its lack of buffering power and varying CO_2 content (which is the chief cause of the charge of inert particles in distilled water) (Abramson, 1934a) prevent reliable comparisons of different suspensions. Any trace of ions brought in with the particles may double or triple the ionic strength and markedly influence mobilities. Unless the ionic strength is known the charge density cannot be calculated (Cf. Abramson, 1934a, 1934b, Abramson and Moyer, 1936, and Moyer and Bull, 1935). Small particles in distilled water have a κr value[3] in the range where deviations in mobility between particles of different size and shape are observed (Abramson, 1931, 1934a, Bull and Moyer, 1936, Kemp, 1935). In biological investigations there is a chance of injury by distilled water which must be considered. Buffers strong enough to eliminate these difficulties and define the ionic conditions are to be preferred. Several workers (Abramson, 1934a) have shown that unless bacteria are washed before investigation they do not give constant results. If the washing is done in distilled water there is a chance that any globulins present in the nutrient medium may be precipitated onto the cell surfaces and hence cause errors.

After rinsing the cell with buffer, the suspension is sucked in through the funnel by means of a mouth pipette and a rubber tube connected to the other end of the cell. Care is taken to eliminate air bubbles. When not in use, the stopcocks are turned so that the electrodes are in contact with the pressure outlet tubes (Abramson and Grossman, 1931) which are filled with saturated Na_2SO_4. To correct for temperature, the data are multiplied by η_t/η_{25} where η is the viscosity of the solution at t° and 25°C (Abramson, 1934a).

Because of electroosmotic streaming of the liquid in the cell, particles must be measured at definite "stationary" levels. For flat cells which are rectangular in cross section, the equation of Komagata (1933),

$$y_{(v_w=0)} = \pm b \sqrt{\frac{1}{3}\left(1 + \frac{384}{\pi^5 k}\right)}, \qquad (3)$$

[3] Where r is the radius and $1/\kappa$, which is a function of the concentration, is the thickness of the diffuse double layer very nearly.

applies, where y is the level measured from the central axis; k is the ratio of half the width[4] to half the thickness, b. It is only when $k > 20$ that the stationary levels lie at 0.21 and 0.79 of the total depth in accordance with the theory of Smoluchowski (Abramson, 1934a). The cells described in this paper are rectangular and fulfill these conditions.

If the "flat" cell is not completely rectangular in cross section, the position of the stationary levels is not known. For this reason, cells produced by flattening a test tube (Northrop and Kunitz, 1925) or those in the form of an ellipse (Enslin, Lindau, and Rhodius, 1935) are not satisfactory. The mere fact that these cells may give a symmetrical parabola of particle mobility with depth is no evidence that the requirements of the Komagata or Smoluchowski equations are being fulfilled. Several investigators have concluded that because integration of Ellis' equation (Abramson, 1934a),

$$V = \frac{1}{x_1} \int_0^{x_1} V_{obs}\, dx \tag{4}$$

(where V is electric mobility of the particle; x_1, the total depth; V_{obs}, the observed mobility at any depth, x), gives a value for V corresponding to 0.21 and 0.79 of the total depth of their cells, therefore these are the correct levels. The parabolas which Komagata has experimentally determined for flat, *narrow*, rectangular cells have been integrated graphically by the author by the use of Simpson's rule to yield a mobility (from equation 4) which falls at the 0.21 and 0.79 levels, yet, as Komagata has shown, these are not the true levels. Equation 3 gives the true mobility in these cases. Of course, if the cell is not even rectangular, equation 3 cannot be used.

The error into which one can fall by improper use of Ellis' equation is shown by the work of Dummett and Bowden (1933). These experimenters used a Mattson cylindrical cell and found that it gave a parabola with depth. They integrated and con-

[4] In an earlier paper (Moyer, 1934b), this was incorrectly stated as length instead of width.

cluded that the true stationary levels lay at 0.21 and 0.79[5] whereas it had been shown by Mooney (1924) and others (Abramson, 1934a) that the depth for $V_w = 0$ in a cylindrical cell lies at the level 0.14.

The cell can be checked for symmetry and flatness by first coating it thoroughly with a one per cent gelatin solution dissolved in distilled water and then measuring the parabola with depth of gelatin-coated quartz particles suspended in 0.1 per cent gelatin (after being coated) in distilled water. Moyer and Abramson (1936) have shown that under these conditions the parabola extrapolates through the origin (i.e., the ratio of electroosmosis to electrophoresis is 1.0). If this does not happen, the cell is unsatisfactory.[6] Abramson (1928) has shown that certain incompletely rectangular cells did not give a ratio of 1.0.

PRECISION AND ACCURACY OF THE INSTRUMENT

A simple experiment was performed to demonstrate the constancy of results on protein-coated particles under suitable conditions. Finely ground quartz particles were suspended for fifteen minutes in 1 per cent Difco gelatin. Meanwhile the cell was also filled with the same solution. This was then diluted to 0.1 per cent gelatin by the addition of acetic acid so that the final acid concentration was M/100. The current was kept constant and the particles were studied for over three-quarters of an hour. During this time, no changes in mobility due to diffusion from the electrodes were noted. Fifty particles were timed at each of the two stationary levels. It could be shown statistically that there was no significant difference between the data from the two levels. Therefore the data were combined to obtain the histogram in figure 4. The range (= 2 S.D.) is from 1.39–1.57 μ/sec./volt/cm. with a mean of 1.48 $\pm$0.004 (calculated from the mean time value). In this experiment, the standard devi-

[5] The parabola obtained in a cylindrical cell by Abramson (1931) can also be integrated in this fashion (which assumes $dV_{obs.}/dy = 0$) to yield similar results.

[6] It is improper to assume that if the cell gives a correct isoelectric point for the protein under these conditions it must be rectangular, for with a protein-coated particle and wall at the isoelectric pH, there is no motion anywhere in the cell (Abramson, 1934a).

ation (S.D.) of a single measurement is 0.044 ±0.003 and the coefficient of variation is 3.0 per cent. These calculations and figure 4 demonstrate the precision to be expected and also show the uniformity of gelatin-coated particles. It is believed that most of the variation in these data resides in errors of timing, focussing, and estimation of the field strength. Although gelatin from a single preparation is very uniform, it is well known that two batches may differ widely; therefore this mean value is not intended as a standard for comparison.

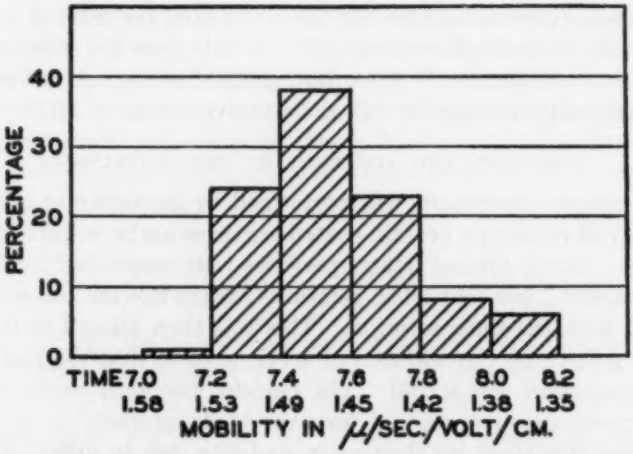

FIG. 4. HISTOGRAM OF MOBILITIES OF GELATIN-COATED QUARTZ PARTICLES IN M/100 ACETIC ACID. ABSCISSA GIVES μ/SEC./VOLT/CM. AND ALSO TIME REQUIRED TO TRAVEL A FIXED DISTANCE.

When bacteria are investigated the situation is somewhat different. Most of the errors are then due to variations in the bacterial surfaces. Some strains are less variable than others. It is well to plot a histogram of a strain of bacteria before using the strain for research. Unimodality and a low coefficient of variation are desirable. Figure 5 shows the histogram obtained by timing 100 cells of the Cohen strain of *Escherichia coli*. This is a smooth laboratory strain of long standing which has been used in many of the growth studies from this laboratory. In peptone, the cells appear to be non-motile. When a culture, grown

on extract agar, was suspended and washed three times in M/50 acetate buffer at pH 5.5 by centrifugation, the mean mobility was 0.519 $\pm 0.006\mu$/sec./volt/cm. The standard deviation of a single observation was 0.056 ± 0.004, with a coefficient of variation of 10.9 per cent. When washed and measured in distilled water, the coefficient was usually around 16 per cent. These data are merely examples of what may be expected with the present technique and should not be used as a standard for reference.

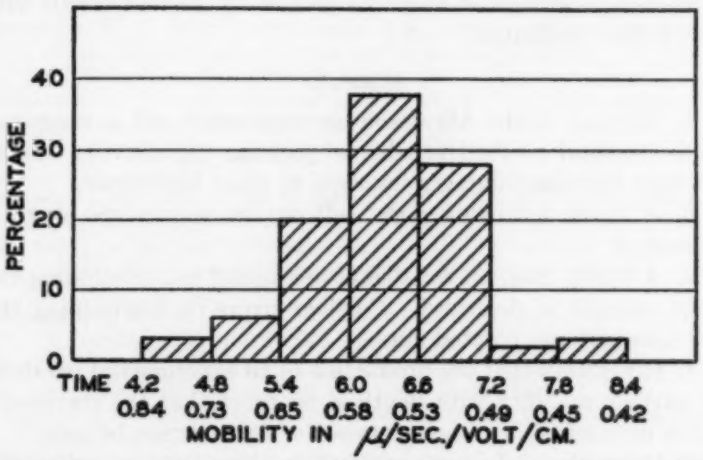

FIG. 5. HISTOGRAM OF MOBILITIES OF ESCHERICHIA COLI FROM A 16-HOUR CULTURE SUSPENDED AND WASHED IN M/50 ACETATE BUFFER AT PH 5.5. ABSCISSA GIVES μ/SEC./VOLT/CM. AND ALSO TIME REQUIRED TO TRAVEL A FIXED DISTANCE.

It will be noted that the variation of gelatin-coated particles is much less (fig. 4).

It is advisable to check the calibration of the cell by some standard particle. Although proteins or latex particles might be used, the most suitable and available particle is the human red blood cell. Abramson (1929b) has found that the human erythrocyte has an exceedingly constant mobility under all sorts of bodily conditions. It does not vary with the blood group, race, age, or sex. He reports a mobility of 1.31 $\pm 0.02\mu$/sec./volt/cm. for the human red cell in M/15 phosphate buffer at pH 7.35. In experi-

ments carried out independently in this laboratory, values from 1.29–1.30 were obtained (for blood from one individual.)

Using a similar instrument of identical central cell construction, the reproducibility of results has been demonstrated with latex particles (Moyer, 1934a, 1934b, 1936) and cholesterol from different preparations (Moyer, 1934c, 1935). In regard to the absolute accuracy of the instrument, the curves of Abramson (1934a) for protein-coated quartz particles coincide almost identically with those obtained on dissolved protein by Tiselius (1930) with the U-tube technique.

SUMMARY

1. The use of the Abramson electrophoresis cell is suggested as a standard method capable of yielding quantitative results without the disadvantages involved in other techniques.

2. A frame for holding the cell on the microscope stage is described.

3. A wiring diagram capable of measuring and controlling the field strength is described. Possible errors in determining the field strength are pointed out.

4. It is shown that the production of an experimental parabola of particle mobility with depth is no proof that the stationary levels determined by Smoluchowski for flat cells can be used.

5. Using the techniques outlined in this communication, the value given by Abramson for the electric mobility of the human erythrocyte has been obtained. The precision of the cell in the investigation of proteins has been tested.

6. It is suggested that the indiscriminate use of distilled water as a suspending medium is not to be recommended.

7. Data are presented to show the amount of variation to be expected in the investigation of quartz, gelatin, and bacterial surfaces.

8. Unless data are accompanied by a statement concerning the construction of the cell used and the method of determining the field strength, results must be accepted with caution.

It is a pleasure to acknowledge the advice and interest of Professor C.-E. A. Winslow, in whose laboratory this work was done.

Thanks are also due to Dr. Harold Mestre, and Dr. H. H. Walker for their suggestions and help.

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THE OXYGEN UPTAKE OF MARINE BACTERIA

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In recent studies from the Woods Hole Oceanographic Institution (Waksman and Carey, 1935a), it was shown that stored sea water rapidly undergoes a decrease in oxygen content, with a somewhat parallel increase in numbers of bacteria up to a maximum population level, after which oxygen decrease continues to take place in the water. The rates of bacterial multiplication and oxygen consumption were considered as indices of the availability of the organic substances present in the water, in solution and in suspension. In order to learn whether the marine bacteria of themselves could account completely for these phenomena, a study was undertaken on 25 pure cultures of organisms freshly isolated from the sea,[2] with the object of determining their rates of oxygen consumption in the "resting" condition, and the influence of temperature as well as of added organic matter of both readily and not so readily utilizable nature on these rates. Glucose, C.P., was selected as probably the most readily available substance, and purified alginic acid[3] as one less generally available. The latter compound occurs abundantly in marine algae (Waksman, Carey and Allen, 1934).

[1] Contribution No. 95 of the Woods Hole Oceanographic Institution.

[2] All cultures were isolated and kindly supplied to the writer by Dr. S. A. Waksman. They are being carried in the collection at Woods Hole for future study, but are referred to here by number, awaiting identification or description. The limited objective of this study, as stated above, renders less important the specific name of each organism. Three of the species however, which were recently described (Waksman *et al.*, 1934), are: *Bacterium alginicum* (AA2); alginic acid bacterium No. 8 isolated from diatom tow and related to *Bacterium alginovorum* (AA8); and, *Bacterium fucicola* (AA17).

[3] Prepared and kindly furnished to the writer by Dr. S. A. Waksman.

In order to obtain data that might be compared with previous investigations of both marine (Pütter, 1924, Waksman and Carey, 1935a) and non-marine organisms (Callow, 1924; Cook and Stevenson, 1928; Harvey, 1928; Martin, 1932; etc.), the amount of nitrogen and in most cases the number of cells[4] in the different suspensions were determined. Since the nitrogen content of the dry weight of many bacterial organisms has been found to be approximately 10 per cent (Nicolle and Alilaire, 1909; Hopkins and Peterson, 1929; Hirsch, 1934), one can (by assuming that this value holds approximately for the species in the present study also) readily arrive at a rough figure for oxygen consumption in terms of dry weight of the cells. Dry weight could not be determined in the present study because most of the organisms cytolyzed readily in distilled water, making adequate washing of the cells for weighing purposes almost impossible.

For each species of bacteria, several Petri plates containing sea-water agar (Waksman, *et al.*, 1933) were heavily inoculated and incubated 24 hours at room temperature. The growth was removed from the surface of the agar by gently brushing it into some sterile sea water with the aid of a camel's hair brush. The cells were centrifuged and washed twice with sterile sea water, and finally suspended in sterile M/4 phosphate buffer,[5] at pH 8. The buffered suspension of cells was aerated with a stream of filtered air for ten to fifteen minutes. Two cubic centimeters of the suspension were then transferred to the usual conical-shaped Warburg respirometer vessels in duplicate, each vessel containing 0.2 cc. of 10 per cent KOH solution in the inset, and the oxygen consumption followed manometrically over a period of from two

[4] All the counts, and most of the preparations for the counts, were made by Dr. Margaret Hotchkiss. The writer is also indebted to Mr. Theone Cordon for assistance in the nitrogen determinations.

[5] Sea water itself was not used as the suspension medium in order to avoid the initial rise in pH resulting from the absorption of CO_2 in the respirometer vessels. Preliminary work on one of the organisms indicated no significant difference in the rate of endogenous O_2-uptake of the cells in phosphate buffer, M/2 NaCl-dilute borate buffer, or sea water-dilute borate buffer. Strong borate (M/4) buffer alone abolished O_2 consumption. The oxidation of glucose went on at a much greater rate in phosphate buffer than in either of the other two suspension media tried.

to four hours. The gas phase in the vessels was air, and the speed and amplitude of shaking were, according to earlier experiments with suspensions of various densities of cells and rates of oxygen consumption, more than sufficient to insure full respiration and complete absorption of CO_2. As soon as possible after placing the manometers on the thermostat, the number of cells per cubic centimeter in the respective suspensions was determined by direct microscopic count of a standard calibrated loopful of suspension spread over an area of 4 sq. cm. on a microscope slide and stained with methylene blue. The number of cells was usually near 10^9 per 1 cc. Macro-Kjehldahl determinations of the nitrogen in 50 cc. (or more) of the same suspensions gave, on the average, a value of about 0.06 mgm. nitrogen per 1 cc. of suspension

The glucose and alginic acid solutions were made up to 0.2 per cent, sterilized by filtration through a Seitz filter, and stored in sterile containers. One milligram (0.5 cc. solution) of the alginic acid was added to the cells from the side arm of the respirometer vessels, after the rate of oxygen uptake without added substrate had been determined. The glucose was added to duplicate vessels at the start of the experiment, and direct comparison made with the rate of oxygen uptake in check vessels containing corresponding amounts of the same suspension of bacteria, but without the substrate.

In the experiments carried out at lower temperatures, the thermostat was controlled by means of a cooling coil of an electric refrigerating unit,[6] whose relay was connected by a vacuum tube relay with a mercurial thermoregulator in the water bath.

The ratio of oxygen uptake to nitrogen content in milligrams, for 25 species of bacteria at 25°C. over a period of two hours, is shown in figure 1. During this period, the decrease in the rate of respiration with time appears to be in most cases slight. The same results calculated in terms of oxygen consumed per 10^9 bacterial cells are illustrated in figure 2. As one would expect, both methods of calculation show considerable differences among

[6] The writer is indebted to Dr. A. C. Redfield and Mr. Ketchum for the loan of this apparatus, and for the assistance of the latter in assembling it.

the different species in the rates of their endogenous O_2 uptake. The distribution is somewhat more uniform in terms of O_2/N than in terms of O_2/cells, since a larger number of species have a rather slow rate of oxygen consumption expressed in terms of oxygen uptake per cell. It seems evident, from the data at hand,

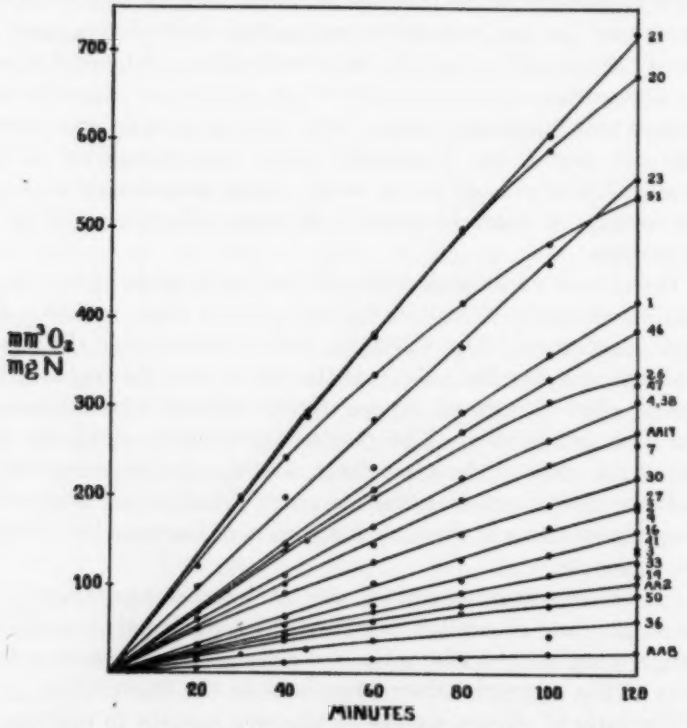

FIG. 1. RATES OF OXYGEN CONSUMPTION, IN TERMS OF O_2 PER MILLIGRAM NITROGEN, BY WASHED CELLS OF 25 SPECIES OF MARINE BACTERIA

that there is no direct relation between the nitrogen content of the cells of different species and their rates of respiration.

A question naturally arises concerning the accuracy with which the number of cells was determined. The results of both the plate counts of viable cells and the direct counts of total cells, just before and following a four-hour period in the respirometers,

are reported in table 1. The plate counts are in fairly good agreement, indicating a slight increase (except for species 1) in the number of cells where glucose was added and a slight decrease without glucose. The direct counts show greater variability.

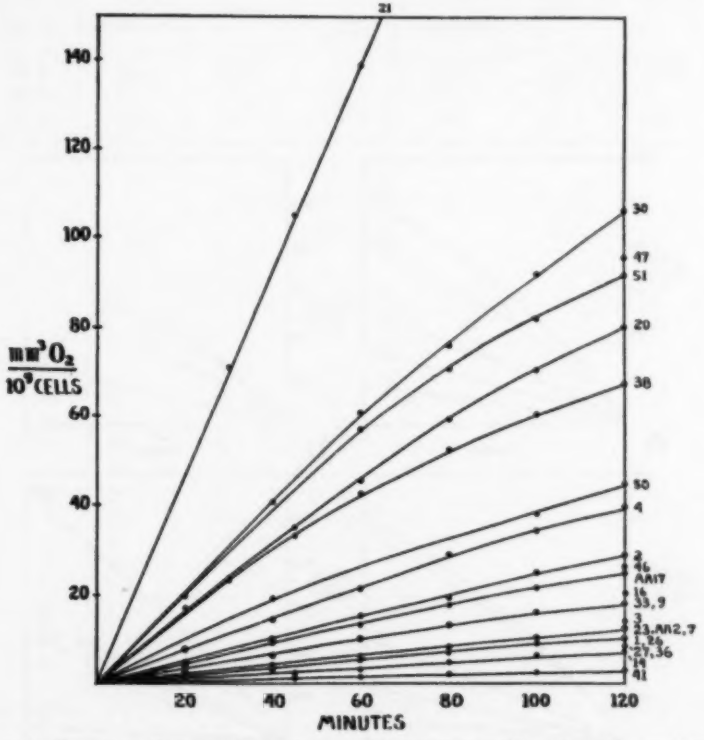

FIG. 2. RATES OF OXYGEN CONSUMPTION, EXPRESSED IN TERMS OF O_2 PER CELL, BY 24 SPECIES OF MARINE BACTERIA

A comparison of the rates of endogenous oxygen uptake by two bacteria, at 5°, 15° and 25°C., is given in figure 3, A and B. Successive experiments, using the same species, were plotted, to show the degree of variation at the different temperatures. Out of a total of 16 experiments, the extreme variation in oxygen uptake per milligram nitrogen amounted to 88 per cent, which apparently

TABLE 1

Comparison of plate counts and direct counts of the cells in three suspensions previous to and following a period of four hours in Warburg respirometer vessels

SUSPENSION OF SPECIES NUMBER	PLATE COUNT: CELLS × 10^9/cc.			DIRECT COUNT: CELLS × 10^9/cc.		
	Before	After 4 hours		Before	After 4 hours	
		No glucose	+ 1 mgm. glucose		No glucose	+ 1 mgm. glucose
AA17	0.65	0.60	0.75	1.05	0.55	0.60
1	1.35	1.20	0.85	0.65	0.70	0.30
21	1.05	1.00	1.30	1.25	0.62	0.40

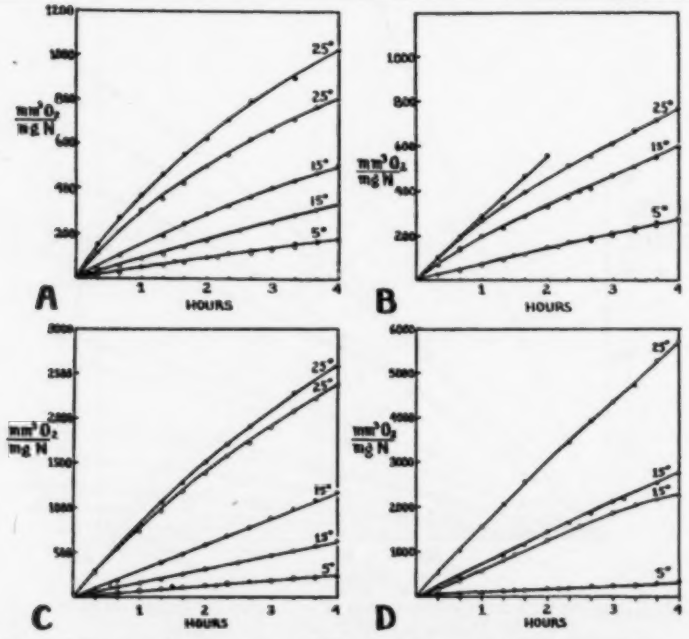

FIG. 3. OXYGEN CONSUMPTION OF WASHED CELLS OF 2 SPECIES OF MARINE BACTERIA, IN TERMS OF O_2 PER MILLIGRAM N, WITH AND WITHOUT ADDED GLUCOSE, AT DIFFERENT TEMPERATURES

A and B, without glucose; C and D, with glucose. Solid and hollow circles represent values obtained in repeated experiments.

is not excessive for bacteria. Callow (1924), for example, found that the variation may amount to as much as 250 per cent, in terms of O_2/milligrams dry weight. In the present study, the

extreme variation did not exceed 200 per cent in terms of O_2/number of cells. The rate of oxygen consumption in the presence of 1 mgm. glucose is illustrated in C and D, figure 3. These may be compared directly with A and B, for they were made on the same suspensions of bacteria. It was found that, as was the case with luminous bacteria (Johnson, 1935), the increase in oxygen consumption due to addition of glucose varied considerably in

TABLE 2

Oxygen consumption of washed cells of marine bacteria with and without added glucose or alginic acid

SPECIES NUMBER	CUBIC MILLIMETERS O_2 PER MILLIGRAM N PER 2 HOURS											
	5°C.			15°C.			25°C.					
	Endogenous	With 1 mgm. glucose	Increase	Endogenous	With 1 mgm. glucose	Increase	Endogenous	With 1 mgm. glucose	Increase	Endogenous	With 1 mgm. alginic acid	Increase
			per cent			*per cent*			*per cent*			*per cent*
23	142	156	10	331	1,417	328	503	3,631	621	557	847	52
21	149	167	12	382	561	46	659	1,546	134	721	778	7.9
AA17	92	126	36	232	446	92	464	1,156	149	271	333	22
1	82	397	378	143	1,141	691	421	2,022	380			
2	39	131	235	164	1,221	644	243	2,864	1,078	190	321	68
4	54	129	138	84	492	485	314	1,830	482	306	350	14
7	85	274	222	164	1,212	639	334	2,637	689	258	405	56
14	89	231	159							112	126	12
41				52	1,733	3,232	74	1,546	1,989	141	182	29
33										136	148	8.8
AA2										104	219	110
AA8										26	31	19
Average..	91	201	148	194	1,028	769	378	2,154	690	256	340	36.2

successive experiments, as well as with different species of organisms. The oxygen consumption of all the bacteria studied, using glucose and sodium alginate as substrates, is summarized in table 2. Only those organisms which gave an increase of more than 5 per cent oxygen uptake over a period of two hours, in the presence of alginic acid, were listed. The great abundance of marine bacteria capable of oxidizing alginate (Waksman, Carey and Allen, 1934) is very obvious. The temperature coefficients

for the endogenous uptake as well as for the oxidation of the glucose are reported in table 3. The unusually high Q_{10} of 9.1, between 5° and 15°C., for the oxidation of glucose by species number 23, seems to suggest the possibility that, for some bacteria, there may be a critical temperature between 5 and 15°, below which glucose is oxidized only very slowly.

The amount of dissolved organic matter in sea water is small, amounting to only around 2.3 grams of carbon in 1 cubic meter (Krogh, 1934), equivalent to 0.00023 per cent carbon, as compared with 0.016 per cent carbon in most of the experiments in the present study. Since glucose appears to be the most readily

TABLE 3

Temperature coefficients for oxygen consumption per milligram N per two hours of 8 species with and without added glucose

SPECIES NUMBER	Q_{10} (ENDOGENOUS)		Q_{10} (+ 1 MGM. GLUCOSE)	
	5° to 15°C.	15° to 25°C.	5° to 15°C.	15° to 25°C.
AA17*	2.5	2.0	3.5	2.5
21*	2.5	1.7	3.3	2.1
23*	2.3	1.5	9.1	2.5
1	1.7	2.9	2.8	1.7
2	4.2	1.4	1.1	2.3
4	1.5	3.7	3.8	3.7
7	1.9	2.0	3.2	2.1
Average.....	2.3	2.18	3.8	2.4

* Average values obtained from 2 or 3 repeated experiments.

utilizable sugar (Kendall and Gross, 1930), it would hardly seem likely that the rate of endogenous oxygen uptake by bacteria in sea water would be increased to any considerable extent by the dissolved organic matter, above the values given in table 2 for the respective species. During active bacterial reproduction, the rates of oxygen consumption might be expected to be higher than during the "resting" periods. In this connection, it is interesting to compare these results with the data of Waksman and Carey (1935b, table VI), on the rate of oxygen consumption and bacterial multiplication in stored sea water. They found that at 22°C., the addition of 10 mgm. glucose per liter (0.001 per cent) caused an increase in total oxygen consumed over a period of two

days (between the first and third) of 197 per cent. The average per cent increase in O_2 uptake by the "resting" cells of the 8 species studied here, at 25°C., was found to be 690 per cent. However, the concentration of glucose was 40 times that used by Waksman and the per cent increase was found to be considerably less at lower concentrations of glucose. Waksman's data show that, between the first and third day, when the bacterial population remained practically stationary, the oxygen consumption per cell (taking the average number of cells over the two day period) per hour amounted to 16.5×10^{-9} mm.,[3] without glucose, and to 18.4×10^{-9} in the sample containing an initial concentration of 0.001 per cent glucose. In comparison with these values, the average value for the endogenous oxygen uptake per cell per hour, over a period of two hours, of 24 species taken at random, was 21.3×10^{-9} mm.,[3] at 25°C.; this is an extraordinarily close agreement, considering that the figures were arrived at by two such widely different methods. Furthermore it is a definite indication that the bacteria alone could easily account for the oxygen consumption in stored sea water, as described by Waksman and Carey (1935a and b).

In the present investigation, no organism was found capable of consuming as much oxygen as 534×10^{-9} mm.[3] per cell per hour, as reported by Pütter (1924) for *Bacterium fluorescens-liquefaciens* in sea water. It is necessary, however, in making comparisons with the rates of O_2 uptake for various bacteria reported by different workers to emphasize that they have not all been obtained by the same procedure; it has also been frequently found that organisms of the same species may vary widely at different times in their rates of respiration.

SUMMARY

The rates of oxygen uptake by washed cells of 25 species of bacteria freshly isolated from the sea were found to vary between 2×10^{-9} and 132×10^{-9} mm.[3] per cell per hours, with an average for all species of 21.3×10^{-9} mm.[3] In terms of milligrams of nitrogen the variation was between 15 and 360 mm.[3] O_2 per hour, with an average of 140, for all species.

The increase in the rate of O_2 uptake in a 0.04 per cent glucose

solution varied for 8 species between 10 and 378 per cent, with an average of 148 per cent, at 5°C.; between 46 and 3232 per cent, with an average of 769 per cent, at 15°C.; and between 134 and 1989 per cent, with an average of 690 per cent, at 25°C. The average temperature coefficients for the endogenous O_2 uptake of 8 species were Q_{10} 2.3, between 5° and 15°C., and 2.18, between 15° and 25°C. For the oxidation of glucose, the average Q_{10} was 3.8, at 5° to 15°C., and 2.4 at 15° to 25°C.

Sodium alginate, in concentration of 0.04 per cent increased the rate of oxygen consumption by the washed cells of 11 out of the 25 species tested, the per cent increase ranging from 7.9 to 110 per cent, with an average of 36.2 per cent.

The writer acknowledges with pleasure and appreciation the generous interest of Dr. Waksman in this work.

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STUDIES ON ANAEROBIC BACTERIA

IX. ANTIGENIC RELATIONS OF CLOSTRIDIUM BIFERMENTANS AND CLOSTRIDIUM CENTROSPOROGENES[1]

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The systematic position, or right to specific rank, of the anaerobic bacillus, *Clostridium centrosporogenes*, has been somewhat insecure, since its conception as *Bacillus centrosporogenes* by Hall in 1922. Originally created to cover 4 strains resembling *Clostridium bifermentans* in many respects, the new species was recorded as actively motile at 8 hours, though non-motile at 24 hours. Deep colonies of *C. centrosporogenes* in meat infusion agar were described as more fluffy and diffuse, but this character was recognized as affected by consistency of the agar. Failure of *C. centrosporogenes* to react in an agglutinative serum for one strain of *C. bifermentans* was also cited. These last points of difference were considered minor and only motility was used in separation of *C. bifermentans* and *C. centrosporogenes* in Hall's key to anaerobes. Robertson (1929) questioned the specific rank of *C. centrosporogenes*, if separation from *C. bifermentans* were to rest upon motility alone.

In considering the position of the new species Hall recognized its physiological likeness to *Clostridium sporogenes* but decided against close affinity because of the central position of its spores and its positive glycerol fermentation. Nevertheless, Kahn (1924) and Weinberg and Ginsbourg (1927) suggested that the new culture was merely a variant of *C. sporogenes*. This possibility was considered by Drake and Sturges (1929) in a study of

[1] This work has been supported in part by a grant from the Wisconsin Alumni Research Foundation.

21 strains of the *bifermentans* group (including certain strains labelled *centrosporogenes*), but they were able to distinguish the entire group from *C. sporogenes* by dimensions of the cells, activity in motility, Gram reaction, hemolysis, fermentation of mannose, conductivity in gelatin media, etc. Differentiation within the *bifermentans*:*centrosporogenes* group by these characters was not justified in their opinion, nor were they able to confirm the separation on the appearance of colonies on blood agar, suggested by Thompson (1926). Various other publications, Kahn (1925); Hall and Whitehead (1927); Quick and Kahn (1929); and Valley (1929), have listed both organisms but, since they were not primarily concerned with species problems, need not be considered here.

Bergey's Manual (1923, 1925, 1930, and 1934) has recognized the two species with separation based mainly upon motility. The same was true of the tentative key of Spray presented in 1933; this, however, was changed in the 1934 key and the two names listed as synonyms, the species being considered as *Clostridium bifermentans* and motile. The Medical Research Committee (1919) and Lehmann-Neumann-Breed (1931) recognized only *Bacillus bifermentans* and that as non-motile. However, Zeissler and Rassfeld (1929) listed *Bacillus putrificus-tenuis* (considered synonymous with *B. bifermentans*) as possessing peritrichiate flagella.

It appears then that the point of most contention is whether *C. bifermentans* is motile or non-motile. Tissier and Martelly (1902) first described it as non-motile but gave no conditions of the test. Hall's acceptance in 1922 and redescription of it as a non-motile organism was apparently based on the fact that his 3 strains had never, to his knowledge, shown motility and were, in fact, non-motile under conditions allowing motility of certain other strains, which as we have seen, he designated *B. centrosporogenes*. It is significant, however, that one of his motile strains had previously been accepted by many as *B. bifermentans*, being the strain No. 506 of the Lister Institute, originally contributed by M. Robertson. The possibility of motility in *C. bifermentans* seems not to have been questioned

again until Sturges and Drake (1928) attempted to sort out their collection of strains of that general group, in comparison with certain type strains of each species furnished by Hall. They noted in some cases a rapid loss of motility during examination and therefore used the capillary tube technique for the protection of delicate anaerobes against aeration during the test of motility. In this way they were able to show motility in Hall's *B. bifermentans* strains as well as his *B. centrosporogenes*. Flagella stains made from young cultures also revealed peritrichiate flagella on cells of both "species", very convincing evidence of which is their excellent plate showing flagella on Hall's "nonmotile" Strain 50 of *B. bifermentans*. Zeissler (1929) also recorded flagellation and motility of certain strains of *B. bifermentans* in the Lister and Pasteur Institutes, although in English and French literature the same strains are reported as nonmotile. Glathe and Cunningham (1933) agreed that the Lister strains No. 506 and No. 2914 (the latter originally Hall's strain 50) are motile, as are also 8 strains which they isolated from farmyard manure. They are inclined to emend the Tissier-Martelly description of *B. bifermentans* to admit motile strains.

The only point at issue then is the reported inagglutinability of *C. centrosporogenes* in a *C. bifermentans* serum and of *C. bifermentans* in one of *C. centrosporogenes* (Hall, 1922). No reaction was found at 1:20 dilution, though no detail of the test is given. Titers of the sera were low, 1:160 and 1:2000, and only 3 strains each were tested. With the more complete analysis of agglutinogens now possible by the technique of flagellar and somatic agglutination, there is reason to retest the cross agglutination and so to put to still another test the identity of the species.

CULTURES AND METHODS USED

Nine cultures were available, several of which are, in fact, strains cited in the papers mentioned above. (See table 1.) The numbers and grouping of the last 7 cultures are those of Drake and Sturges (1929). After the routine testing of the cultures as to purity, observations were made upon their motility by the capillary tube method of Sturges and Drake. In 12-

hour cultures in liver-infusion broth motility was observed in all cases, Sturges' Nos. 2 and 3 and Lister 506 being least active. On the basis therefore of these observations and with regard for the key cultures, historically speaking, the following were chosen for production of sera: Lister *bifermentans* 506, Hall's *centrosporogenes* 76, and Sturges' *bifermentans* 2 (= Hall's 50).

Antigens for injection and later tests were produced as surface growth on liver extract agar of pH 7.6, enriched with 0.1 per

TABLE 1

Cultures used

NAME AS RECEIVED	SOURCE	INITIAL INFORMATION
B. bifermentans Strain 506	Lister Institute	Motile at 8 hours; not at 24 hours. Hall, 1922
B. centrosporogenes Hall strain 76	American Type Culture	One of the original strains of the species; motile. Hall, 1922
C. bifermentans	W. S. Sturges	Hall considered non-motile. Flagella and motility demonstrated by Sturges and Drake, 1928
Strain 2 = Hall's Strain 50		
Strain 3 = Hall's Strain 70		
Strain 5 = Hall's Centro—408 C	W. S. Sturges	Motility easily demonstrated by Hall, and by Sturges and Drake
Strain 8 = Hall's Centro—472		
Strain 12 Sturges	W. S. Sturges	Motility easily demonstrated but "other characters intermediate between above groups"
Strain 13 Sturges		
Strain 6 = Hall's Centro 76	W. S. Sturges	Motility easily demonstrated. Sorbitol fermented

cent glucose and 0.5 per cent bacto tryptone. Three-day cultures yielded a moderate crop of cells, purely vegetative, which were collected with one washing by centrifugation.

As in previous work of this kind (McCoy and McClung, 1935) sera were produced by injection of living cells (so-called whole-cell or W condition) and by injection of cells heat-killed by 2 hours' steaming (so-called somatic or O condition). Dosage and intervals of injection were substantially as in the former paper. Collection of sera and their preservation by refrigeration alone

were also similar, but the details of setting up and incubating for the macroscopic tube tests with these organisms were changed somewhat from our former practice. Incubation at 54°C. for 6 to 8 hours, followed by refrigeration overnight was used after comparison of tests run under various other conditions, completeness of flocculation with sharp end point at titer being considered. Some advantage in stability of the test antigen through the long incubation was found in the use of 0.4 per cent instead of the usual 0.85 per cent saline. The technique for absorption of agglutinins was essentially that of our former publications.

EXPERIMENTAL RESULTS

Preliminary tests of the cross reactions of all 9 cultures showed definite reaction in both W and O sera (tables 2 and 3). Flocculi were delicate and in most cases one would hesitate to claim presence of an H (labilotropic) factor upon appearance of flocculation alone. As more convincing demonstration of the respective O (stabilotropic) and H elements in the antigen and their corresponding agglutinins in the sera, a series of absorption tests was undertaken. For simplicity of presentation, results will be given for only *bifermentans* 506, *centrosporogenes* 76, and *bifermentans* 2, for which complete reciprocal crosses can be given. It should be emphasized, however, that in every test, whether of direct agglutination, absorption, or test after absorption, the additional 6 organisms were used in one-way cross reactions. In no case was there reason to question their behavior, nor to suppose that among them was any which would not fall within the behavior of the 3 strains, whose reactions are completely known.

Distribution of the H factor among the three strains. In analysis of agglutinogens by the H-O technique, the proof of presence and identity of the H factor between strains of a species is first to be considered. Indeed in the case of *bifermentans*:*centrosporogenes* proof of possession of the same H factor by both organisms is of peculiar interest because of the supposed relation of H antigen to motility. Analyzed for H or so-called flagellar factor the agglutinative relations of the chosen strains are set

out in table 4. Our *bifermentans* 506 and *centrosporogenes* 76 undoubtedly possess the same H antigen, as shown by ability: to stimulate production of H agglutinins in the animal body, to absorb such agglutinins from either serum, and to react *in vitro* with delicate but typical H flocculation in a purified H antiserum

TABLE 2

Cross agglutination of 9 strains of C. centrosporogenes: C. bifermentans in W sera*

TEST ANTIGEN	W SERA FOR		
	Centr 76	Bi 506	Bi 2
W antigen			
Centr 76	**2560**	1280	5120
Bi 506	1280	**2560**	10240
Bi 2	2560	640	**10240**
Bi 3	1280	1280	5120
Bi 5	1280	2560	5120
Bi 6	5120	5120	10240
Bi 8	1280	2560	10240
Bi 12	2560	320	5120
Bi 13	1280	640	10240
O antigen			
Centr 76	5120	5120	2560
Bi 506	5120	5120	5120
Bi 2	2560	5120	5120
Bi 3	1280	5120	2560
Bi 5	2560	1280	5120
Bi 6	2560	5120	5120
Bi 8	1280	2560	2560
Bi 12	1280	2560	2560
Bi 13	1280	2560	2560

Homologous titers are set in boldfaced type.
* Whole-cell.

(*i.e.*, a W serum absorbed to virtually complete removal of O agglutinins). The *bifermentans* 2 of Sturges, which it will be remembered is the so-called non-motile Strain 50 of Hall, fulfills the first two requirements, thus demonstrating its H-agglutinogenic and H-absorptive capacity. Its ability to flocculate in purified H antiserum is variable: slight in sera of *centrosporogenes*

76 and *bifermentans* 506 to considerable in the homologous serum, all O-absorbed to −40 homologous titer. Perhaps this difference corresponds to chance variation in degree of motility of the particular cultures taken for antigen, though no records were kept on this point. Another factor may be the greater potency of the *bifermentans* 2 serum, in this particular case. At any rate what difference there may be between *bifermentans* 2 and *centrosporogenes* 76 and *bifermentans* 506 would seem quantitative rather than qualitative. Incidentally, other cases of such H variation were found: Strain 3 of Sturges (= Hall's 70) behaved

TABLE 3

Cross agglutination of 9 strains of C. centrosporogenes: C. bifermentans in O sera*

O antigen

TEST ANTIGEN	O SERA FOR		
	Centr 76	Bi 506	Bi 2
Centr 76	**20480**	10240	2560
Bi 506	10240	**10240**	5120
Bi 2	5120	10240	**2560**
Bi 3	5120	2560	5120
Bi 5	5120	5120	2560
Bi 6	5120	2560	2560
Bi 8	10240	5120	5120
Bi 12	5120	5120	2560
Bi 13	5120	5120	2560

Homologous titers are set in boldfaced type.

* Somatic.

as Strain 2 and Strains 12 and 13 slightly so, in the evidence of variable H development in different batches of antigen, as shown by flocculation.

Somatic agglutination. Possession of a common somatic antigen by the 3 strains is shown clearly in table 5, in which complete reciprocal absorption of somatic agglutinins is evident. And it is further indicated that only one somatic factor is involved; note the virtually complete removal in all cases, whether by homologous or heterologous absorptions. One may assume therefore complete identity of the somatic antigen, not simply the presence of a group somatic factor, as has been reported

TABLE 4

Tests of presence and identity of H factor in C. centrosporogenes 76, C. bifermentans 506 and C. bifermentans 2 by reciprocal absorption experiments

SERUM (W)	ABSORBING AGENT	TEST ANTIGEN (W)	TITER	
			W absorption (Before → after)	O absorption (Before → after)
Centr 76	Centr 76	Centr 76	2560 → −80*	2560 → 2560
		Bi 506	1280 → −80	1280 → 1280
		Bi 2	2560 → −80	2560 → −160
	Bi 506	Centr 76	2560 → −80	2560 → 2560
		Bi 506	1280 → −80	1280 → 1280
		Bi 2	2560 → −80	2560 → −160
	Bi 2	Centr 76	2560 → −80	2560 → 1280
		Bi 506	1280 → −80	1280 → 2560
		Bi 2	2560 → −80	2560 → −160
Bi 506	Centr 76	Centr 76	1280 → −80	1280 → 640
		Bi 506	2560 → −80	2560 → 5120
		Bi 2	640 → −80	640 → 160
	Bi 506	Centr 76	1280 → −80	1280 → 2560
		Bi 506	2560 → −80	2560 → 1280
		Bi 2	640 → −80	640 → 320
	Bi 2	Centr 76	1280 → −80	1280 → 1280
		Bi 506	2560 → −80	2560 → 640
		Bi 2	640 → −80	640 → 320
Bi 2	Centr 76	Centr 76	5120 → −80	5120 → 10240
		Bi 506	10240 → −80	10240 → 5120
		Bi 2	10240 → −80	10240 → 5120
	Bi 506	Centr 76	5120 → −80	5120 → 10240
		Bi 506	10240 → −80	10240 → 5120
		Bi 2	10240 → −80	10240 → 1280
	Bi 2	Centr 76	5120 → −80	5120 → 5120
		Bi 506	10240 → −80	10240 → 5120
		Bi 2	10240 → −80	10240 → 5120

* −80 or −160 indicates no agglutination at these dilutions, the lowest tested in the particular series.

TABLE 5

Proof of identity of somatic antigen by reciprocal absorptions between C. centrosporogenes 76, C. bifermentans 506, and C. bifermentans 2

SERUM (O)	ABSORBING AGENT	TEST ANTIGEN (O)	TITER	
			Before absorption	After absorption
Centr 76	Centr 76	Centr 76	20480	−40*
		Bi 506	10240	−40
		Bi 2	5120	−40
	Bi 506	Centr 76	20480	−40
		Bi 506	10240	−40
		Bi 2	5120	−40
	Bi 2	Centr 76	20480	−40
		Bi 506	10240	−40
		Bi 2	5120	−40
Bi 506	Centr 76	Centr 76	10240	−80
		Bi 506	10240	−80
		Bi 2	10240	−80
	Bi 506	Centr 76	10240	−80
		Bi 506	10240	−80
		Bi 2	10240	−80
	Bi 2	Centr 76	10240	−80
		Bi 506	10240	−80
		Bi 2	10240	−80
Bi 2	Centr 76	Centr 76	2560	−40
		Bi 506	5120	−40
		Bi 2	2560	−40
	Bi 506	Centr 76	2560	−40
		Bi 506	5120	−40
		Bi 2	2560	−40
	Bi 2	Centr 76	2560	−40
		Bi 506	5120	−40
		Bi 2	2560	−40

* −40 or −80 indicates no agglutination at these dilutions, the lowest tested in the particular series.

occasionally. Cross reaction to titer, and as often to one tube beyond homologous titer (*e.g.*, in the cross tests of *centrosporogenes* 76 and *bifermentans* 506 *before* absorption) would also suggest identity rather than group reaction involving a somatic factor.

It will be remembered that in tables 2 and 3 there was presented evidence of somatic reaction in the direct cross tests between all strains studied. In those tests also the reaction occurred in relatively high dilution, approaching or exceeding homologous titer in many cases. This point is worthy of emphasis, because it is sometimes found that in a mixed H-O serum produced for a motile organism, the O or somatic agglutinins are subordinate (Savage and White, 1925). It is suggested in such cases that the H or flagellar antigen is species-specific and the O often group-specific. In other cases, of completely non-motile organisms, such specificity as there may be in the agglutination reaction lies within somatic factors. Specificity of antigens, however, may be well developed; indeed in groups like the streptococci, extreme strain specificity may be found (Andrewes and Christie, 1932). The condition of *bifermentans*: *centrosporogenes* strains is in effect intermediate. H factors are present as we have seen, but they are not dominant and easily differentiated in direct cross tests. This condition presents an interesting transition of antigenic specificity, inasmuch as by their weak and variable motility the organisms also are indeterminate.

C. bifermentans versus C. centrosporogenes

As to the species relation of the 9 strains studied, there seems no question. The differences reported are quantitative rather than qualitative and are, in fact, no greater than have been met in previous studies of strains within a species. In the present case, the evidence of the presence of H factor, though in less amount, in certain of the *C. bifermentans* strains is particularly interesting, since it correlates well with their weaker motility. Thus, the serological data are in good accord with Drake and Sturges' conclusion that there is no consistent nor exclusive

difference between the strains labelled *C. bifermentans* and *C. centrosporogenes*, and that there is therefore no valid reason for recognizing *C. centrosporogenes*. Further, the evidence of H factor in *C. bifermentans* supports their observation that the species is motile, and with this emendation the original description is valid and sufficient.

SUMMARY

Identity of both H and O factors in the antigenic complex of *Clostridium centrosporogenes* 76, *Clostridium bifermentans* 506, and *Clostridium bifermentans* 2 has been shown by reciprocal agglutinin absorption experiments. Tests of 6 other strains in these sera show every probability of their having the same agglutinogens, although the evidence is incomplete.

Presence of the H factor in *Clostridium bifermentans* is consistent with its motility and presence of flagella, as was shown by Sturges and Drake in 1928.

This serological evidence, coupled with the failure of Drake and Sturges and others to find valid morphological and physiological differences between strains of so-called *Clostridium bifermentans* and *Clostridium centrosporogenes*, would indicate that the concept of a single species, *Clostridium bifermentans*, is adequate.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARYLAND BRANCH

BALTIMORE, FEBRUARY 11, 1936

AN APPARENTLY NEW TYPE OF INTESTINAL GRAM-NEGATIVE ROD ANTIGENICALLY RELATED TO BOTH TYPHOID AND DYSENTERY ORGANISMS. *Grace P. Kerby*, Biological Division, Johns Hopkins Hospital, and *Einar Leifson*, Department of Pathology and Bacteriology, Johns Hopkins University.

In October, 1935, M. I. C., 39-year old rancher, entered the Johns Hopkins Hospital for an operation on the left knee. Two days after operation there was an onset of diarrhea which continued for one month. During this time the clinical picture was typical of dysentery, and during the first week the organism here described was repeatedly isolated from stool culture. It is a non-motile, intestinal Gram-negative rod which appears to dissociate rather readily into rough and smooth strains. Its cultural characteristics are those of *Shigella dysenteriae* Flexner.

Preliminary titrations seem to indicate that the organism possesses the Group III "O" antigen of the typhoid group but no flagellar antigen. In the dysentery group it appears to correspond most closely antigenically to Flexner W. The patient's serum, tested throughout the course of the present illness, showed a definite rise and then fall of titre for his own organism and for stock antigens of *Eberthella typhosa* and *Shigella dysenteriae* Flexner.

A SODIUM SELENITE ENRICHMENT MEDIUM FOR THE ISOLATION OF TYPHOID BACILLI. *Einar Leifson*, Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore, Maryland.

The relative number of typhoid bacilli in feces from typhoid patients, convalescents and carriers is often so small that it is practically impossible to find them by direct streaking on plates. This is often the case even with the newer media such as Desoxycholate-Citrate Agar and Wilson-Blair Agar which inhibit the growth of a large proportion of the colon bacilli, and on which the colonies of the typhoid bacilli are readily recognized. Various enrichment media have been used for this purpose but none seem to have been very promising. An enrichment medium containing sodium selenite has been found very successful. On this medium the typhoid bacilli grow very rapidly while the colon bacilli either remain constant, or decrease in numbers. Pyocyaneus and Proteus bacilli grow much better than the colon bacilli and if present in large numbers may interfere with the function of the medium. The medium has been tried on about 100 stools from patients and carriers by the author and by various bacteriologists in Baltimore and a considerably larger number of positive isolations were obtained than by direct streak. Selenite enrichment media are

also being developed for the isolation of typhoid bacilli from oysters, milk, sewage and water.

THE SENSITIZING ACTION OF STAPHYLOCOCCUS TOXIN. *Earl L. Berkey*, Wilmer Clinic, The Johns Hopkins Hospital, Baltimore, Maryland.

Staphylococcus toxin, when injected intracutaneously into rabbits once a week, produces a high degree of immunity to the toxin and to living cultures of staphylococci. Coincident with the development of the immunity the animal becomes sensitive to the broth in which the toxin was produced. If lens protein is added to the medium in which the toxin is produced, the rabbit becomes sensitive to this substance. When the lenses of such lens-sensitive animals are needled, a marked intraocular inflammation of an allergic type develops. This reaction resembles, clinically and histologically, entophthalmitis phacoanaphylactica in humans.

EASTERN PENNSYLVANIA CHAPTER

UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA,

MARCH 24, 1936

PHAGOCYTIC ACTIVITY OF CIRCULATING CELLS IN LEUKEMIAS. *Max M. Strumia and Frederick Boerner*, The Bryn Mawr Hospital, Clinical Laboratory, Bryn Mawr, Pa.

Experiments on phagocytosis employing leucocytes circulating in normal subjects as well as in forms of acute and chronic leukemias, and in the so-called glandular fever (acute mononucleosis) gave the following results:

Normal blood: maximal phagocytic activity is displayed by polymorphonuclear neutrophiles followed closely by monocytes. Eosinophiles also have distinct phagocytic activity but less than that of previous groups. Lymphocytic cells display no phagocytic activity whatever.

Lymphocytic cells of chronic lymphatic leukemias and acute lymphatic leukemias (lymphoblasts, prolymphocytes, lymphocytes and leucocytoid lymphocytes) show no phagocytosis. Oxidase positive hemocytoblasts found in acute granulocytic leukemias showed doubtful or no phagocytosis. In chronic myelogenous leukemias myeloblasts showed very slight phagocytosis and the promyelocytes slightly more. A very sharp increase in the phagocytic activity occurs with the myelocytes and increases to a maximum with the neutrophilic polynuclear leucocytes. This appears to be coincidental with the development of the specific mature neutrophilic granulations. In acute hemohistioblastic (monoblastic) leukemias the large undifferentiated cells (hemohistioblasts or reticulo-endothelial cells) show active phagocytosis. The typical cells occurring in glandular fever (acute mononucleosis) often erroneously interpreted as monocytes, but actually young degenerated lymphocytic cells, show no phagocytosis.

THE EFFECT OF SONIC VIBRATIONS ON (A) PURE PROTEINS, AND (B) BACTERIAL CONSTITUENTS. *Earl W. Flosdorf and Leslie A. Chambers*, Departments of Bacteriology and Pediatrics, and the Johnson Foundation for Medical Physics, School of Medicine, University of Pennsylvania.

With apparatus previously described

(Chambers and Gaines, Jour. Cell. and Comp. Physiol., 1932, **1**, 450–473) some proteins have been denatured to yield products similar to those produced thermally. Other proteins are unaffected (Chambers and Flosdorf, Jour. Biol. Chem., In press, 1936). In spite of the similarity the *mechanism* responsible for chemical action in general, when it does occur, seems *not* to be a thermal one (Flosdorf, Chambers, and Malisoff, Jour. Amer. Chem. Soc., In press).

A method has been developed (to be described in detail by Chambers and Flosdorf) using similar apparatus for the extraction from bacteria of labile substances which are destroyed by ordinary extraction involving heat or chemicals. From *Eberthella typhosa* we have obtained a soluble substance which will absorb from immune serum the antibody against Felix's Vi antigen (with Mr. Richard Greaves). From *Streptococcus hemolyticus* (virulent mucoid) we have obtained a new soluble protein which is altered by heating for 30 minutes at 56° and which if unheated will absorb from immune serum the antibody responsible for phagocytosis (Mudd, Czarnetzky, Pettit and Lackman). The immunological properties of these materials are being investigated further.

THE PHAGOCYTOSIS OF STREPTOCOCCI BY THE NEUTROPHILES IN WHOLE HUMAN BLOOD. *David Lackman, Fred Boerner and Stuart Mudd*, University of Pennsylvania School of Medicine, Philadelphia, Pa.

ANTIGENIC COMPOSITION AND IMMUNOLOGICAL BEHAVIOR OF HEMOLYTIC STREPTOCOCCI—A PROGRESS REPORT. *Stuart Mudd, E. J. Czarnetzky, Horace Pettit and David Lackman.*

Chemical methods involved are: (1) extraction and fractionation of the mucoid material; (2) extraction of bacterial "M" and "C" substances; (3) liberation of antigens from the bacterial bodies by the method of Chambers and Flosdorf.

The serological methods used are: (1) precipitation and agglutination; (2) phagocytosis by washed exudative rabbit leucocytes mixed with the bacteria in serial dilutions of rabbit immune serum; (3) mouse protection. Phagocytosis affords a sensitive, accurate means for titration of sera; the results run parallel to those of mouse protection.

The phagocytosis-promoting and mouse-protective action of antistreptococcal rabbit sera have proven to be type-specific, although with unabsorbed sera minor cross reactions do occur. Contrary to expectation, absorptions of antisera with homologous "M" and "C" substance have not reduced, but have rather increased their phagocytosis-promoting action; this increase in tropic power is associated with the removal of non-specific inhibiting factors in the sera which we suspect may be serum lipoids. Phagocytosis-promoting action of the antisera is, however, completely removed by absorption with a heat-labile substance present in solution after sonic disintegration of the bacteria.

SOME OBSERVATIONS AND CORRELATIONS IN THE DISSOCIATION OF THE DIPHTHERIA BACILLUS. *Harry E. Morton.*

Diphtheria bacilli, representing clinical and laboratory strains, have been dissociated into S (smooth), SR (intermediate), R (ultimate rough) and D (dwarf) colony types. Organisms con-

tained in all of these colony types were true diphtheria bacilli in that they showed the same fermentation reactions (acid in glucose and dextrin, no action in sucrose), produced diphtheria toxin, were pathogenic for guinea pigs and cross-agglutinated with sera produced by immunizing rabbits with any of the other colony types. With these various colony types could be correlated such properties as the manner of growth in broth, stability in saline solutions of varying concentrations of NaCl, appearance on potassium tellurite chocolate agar, hemolytic power, cell morphology and resistance to aging. Dissociation was brought about by altering the pH of the medium, aging accompanied with evaporation of the culture medium and by the presence of LiCl in the medium.

EASTERN NEW YORK BRANCH

STATE DEPARTMENT OF HEALTH LABORATORIES, ALBANY, N. Y.,

MARCH 28, 1936

THE PREDOMINATING STREPTOCOCCI IN NORMAL HUMAN THROATS. *C. E. Safford and J. M. Sherman*, Cornell University and the New York State Department of Agriculture and Markets.

Isolations made by the application of quantitative methods to freshly taken swab samples so as to eliminate organisms of fortuitous occurrence and even true throat streptococci which may occur in small numbers, showed not a single true beta hemolytic streptococcus among 322 cultures isolated at various times from 20 individuals.

The typical throat streptococcus, represented by 290 cultures, was found to have the following characteristics: On blood agar the reaction varies from gamma to alpha, "weak alpha" being characteristic; in glucose broth a final pH of 4.4 to 4.0; litmus milk promptly acidulated and curdled with marked though not quite complete reduction after but not before curdling; no growth at 10°C. nor at 47°C., maximum temperature of growth about 45°C.; no pathogenicity for rabbits by 200 intravenous injection nor for white mice intraperitoneally; sodium hippurate and starch not hydrolyzed; gelatin not liquefied; ammonia not produced from peptone; organisms completely destroyed by a heat treatment of 30 minutes at 60°C. in milk; glucose, maltose, lactose, and sucrose fermented; glycerol and mannitol not fermented; raffinose (93 per cent), inulin (80 per cent), and salicin (95 per cent) are usually fermented.

A separation of the strains which failed to ferment raffinose or inulin into distinct species does not seem justified. If this view is sound, the names *Streptococcus mitis* and *Streptococcus salivarius* (Andrews and Horder, 1906) are probably invalid, and the name *Streptococcus mitior* (Schotmüller, 1903) would appear to be more appropriate.

THE BACTERICIDAL ACTION OF BLOOD. *Nicholas A. Milone*, Bureau of Milk Sanitation, State Department of Health, Albany, New York.

Using *Escherichia coli* as test organism: 4-hour cultures are markedly less resistant than 24- and 72-hour cultures when immersed in relatively fresh whole blood or serum of the horse, cow, sheep and pig.

Progressive loss of bactericidal action occurs when serum is aged at 10°C. and 37°C. but much more rapidly at the latter temperature. pH increases

most rapidly at 37°C. Four-hour cultures, after a period of inhibition or death enter a definite growth phase while 24 and 72-hour cultures give similar results but to a much lesser degree.

Whole blood and serum have similar germicidal powers. In hemoglobin, however, after an initial fluctuation subsequent growth results, with 4-hour cultures eventually showing the most pronounced increase.

Some exhaustion of germicidal substance occurs when the same serum is reinoculated and the test period extended.

Serum filtered through a Berkefeld filter loses some of its lethal action.

Bubbling CO_2 through serum changes the pH from 7.54 to 6.48. When N_2 is used change is from 7.54 to 8.66. Such changes in pH in either direction from normal, result in decreased germicidal action.

Values of K are rarely constant. Survivorship curves point to heterogeneity of bacterial cultures. Results do not uphold the monomolecular theory of death.

SOIL MICROÖRGANISMS WHICH DECOMPOSE THE CARBOHYDRATES OF THE PNEUMOCOCCUS. REPORT ON THE ISOLATION OF TWO NEW STRAINS. *Grace M. Sickles and Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

The isolation of two new strains of soil microörganisms which decompose the specific carbohydrates of the pneumococcus is reported.

One strain, isolated from rich garden soil, actively decomposes type II pneumococcus carbohydrate. It closely resembles *Saccharobacterium ovale*, previously described, except in pigment production. The colonies are yellow; those of *S. ovale* are pink. This microorganism also destroys to a certain extent the precipitating activity of the carbohydrates of pneumococcus type III and type VIII and the nontype-specific carbohydrate of a degraded type I pneumococcus.

The other strain, isolated from uncultivated soil, readily decomposes the carbohydrate of pneumococcus type VIII. It is a narrow pointed rod often crescent shaped, resembling *Saccharobacterium acuminatum* rather than the strains of *Bacillus palustris* previously found active on type VIII carbohydrate. Like *S. acuminatum*, it fails to develop on most of the standard media but does grow slowly on blood agar. In its fermentation reactions it has a much wider range than *S. acuminatum* since in mineral medium A containing carbohydrates in 1 per cent concentration, it utilizes glucose, lactose, sucrose, maltose, dextrin, xylose, and galactose. No gas and very little acid are produced in the decomposition of these carbohydrates by the microorganism. An active cell-free enzyme was not obtained from either strain.

A STUDY OF THE ULTRAFILTRATION AND OF THE ADSORPTION ON CALCIUM PHOSPHATE OF DIPHTHERIA, STREPTOCOCCUS, TETANUS, AND BOTULINUS TOXINS. *James J. Quigley and Gretchen R. Sickles*, Division of Laboratories and Research, New York State Department of Health, Albany.

Our studies of the ultrafiltration of diphtheria and streptococcus toxins indicate wide differences in the molecular structure of the toxins themselves or of the fraction of the toxin broth associated with them. A similar study of tetanus and of types A and B botulinus toxins was undertaken.

Aliquot portions of each toxin were ultrafiltered and washed on a 9.5 per cent nitrocellulose membrane in the Bechhold apparatus, and also ad-

sorbed on calcium phosphate. The diphtheria, tetanus, and botulinus toxins did not pass this 9.5 per cent membrane, whereas approximately 50 per cent of the streptococcus toxin was found in the filtrate. One hundred per cent of the diphtheria toxin and 80 per cent of the type-B botulinus toxin were recovered in the washed residues. Losses in the tetanus and type A botulinus toxins were apparently due to adsorption on the collodion.

The diphtheria, tetanus, and botulinus toxins were adsorbed from the toxin broth on calcium phosphate by the method previously reported. Streptococcus toxin was only partially adsorbed. Diphtheria and tetanus toxins were successfully eluted with 2 per cent of disodium phosphate; a more satisfactory recovery of the botulinus toxins was accomplished by dissolving the precipitate with diammonium citrate instead of elution.

REDUCTION OF VITAMIN C IN THE ADRENALS OF GUINEA PIGS AFTER INJECTION OF DIPHTHERIA TOXIN. PRELIMINARY REPORT. *Calvin C. Torrance*, Division of Laboratories and Research, New York State Department of Health, Albany.

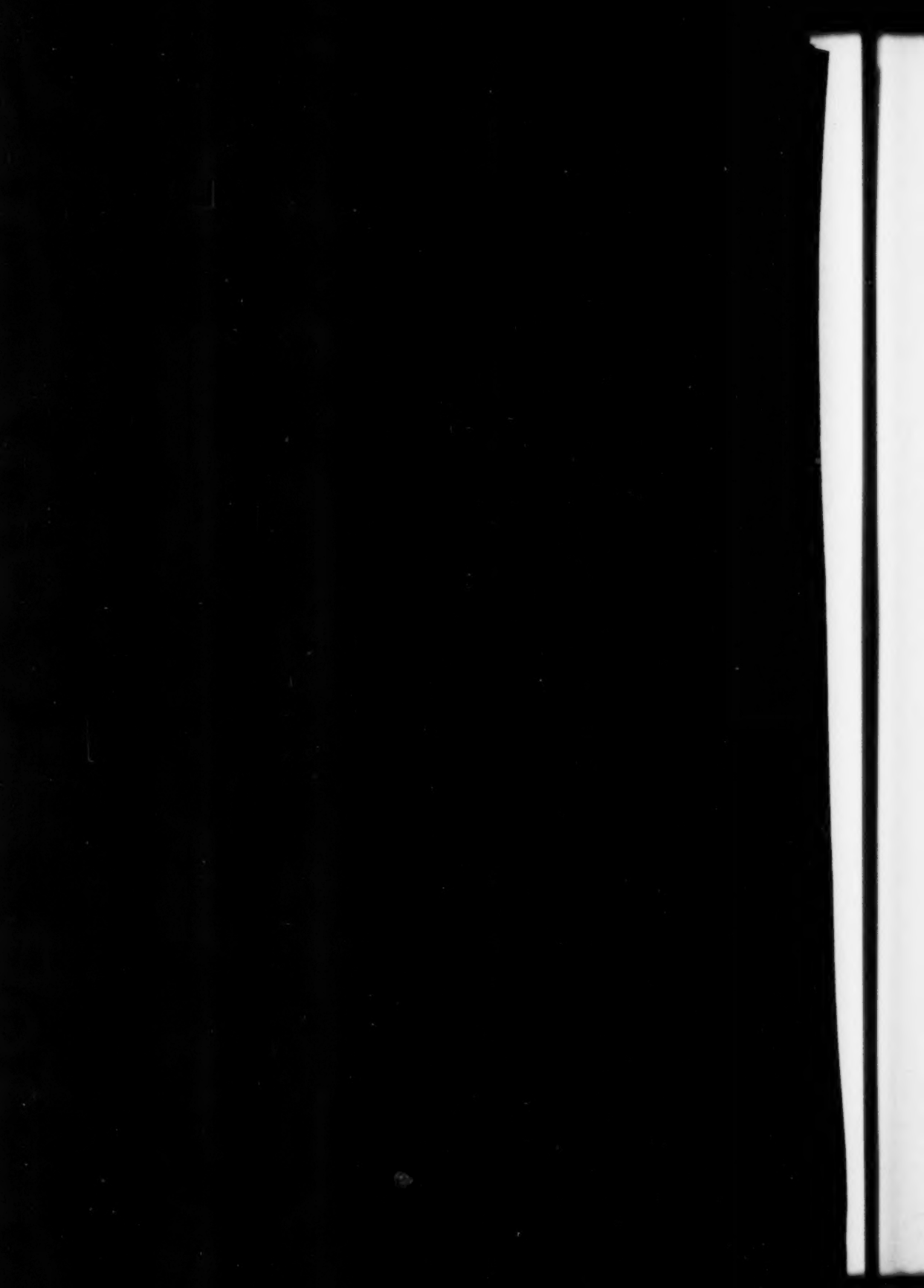
Ten laboratory-bred guinea pigs, weighing from 230 to 280 grams, were each injected in December with a uniform lethal dose of diphtheria toxin; another ten, with half the dose. As each of the former animals died, one of the latter and an uninjected control were destroyed and the vitamin C content of the adrenals determined by the method of Bessey and King. The average vitamin C content of the adrenals of the ten animals which died from the effect of diphtheria toxin was found to be 0.01207 of a milligram, or less than 15 per cent of that for the control animals. The adrenals of five of the animals that received a sublethal dose of toxin and were destroyed approximately 48 hours after the injection showed little change in vitamin C content. Assay of the adrenals of the five remaining animals destroyed after 70 hours gave a figure less than 40 per cent of that for the normal animals. Thus, marked decreases occurred in the vitamin C content of the guinea pigs' adrenals following both lethal and sublethal injections of diphtheria toxin. The decrease was more rapid, however, with the larger doses.

THE SIGNIFICANCE OF THE ESCHERICHIA-AEROBACTER GROUP IN PASTEURIZED MILK. *W. F. Alexander and Nicholas A. Milone*, New York State Department of Health.

The results secured since early 1932 indicate that the colon test cannot be used as an index of the "efficiency of pasteurization" if "efficiency" means a time-temperature relationship. If the term is to include those sanitary factors that are involved in producing and processing a raw milk supply so as to obtain clean and safe pasteurized milk, then the presence of these bacteria therein may have a sanitary and public health significance.

Depending on the media and amount of inoculum used, it was found that 2 to 53 per cent of the raw milk; 83 to 97 and 98 to 100 per cent of the preheated and pasteurized milk in the pasteurizer, respectively; 40 to 70 per cent of the bottled or canned pasteurized milk, failed to show presumptive evidence of this group.

Therefore in 98 to 100 per cent of the cases, when this group of bacteria was found in pasteurized milk in the 0.1, 1.0, or 10 cc. inoculums, it was because of insufficient sterilization of equipment or other undesirable or inadequate methods of pasteurizing plant management and operation.



THE USE OF SEMI-SOLID AGAR FOR THE DETECTION OF BACTERIAL MOTILITY[1]

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Although the hanging-drop method for the detection of bacterial motility can be employed successfully, the procedure has several distinct disadvantages. It is so tedious that the determination of this bacterial characteristic is frequently neglected in the routine laboratory. Furthermore, the results are often uncertain because it is difficult to observe motility when only a few of the cells in a culture are motile. Finally, it is necessary to provide relatively young cultures for the examination.

The use of semi-solid media for the determination of bacterial motility, on the other hand, eliminates the shortcomings of a hanging-drop technique. The results are macroscopic. They are cumulative, thereby particularly qualifying the method for use in the routine laboratory, where examinations cannot always be carried out at a specific time. Moreover, this method practically eliminates the possibility of overlooking motility when only a small proportion of motile cells are present, because the localized out-growths, which occur wherever motile cells are deposited along the stab, can hardly escape notice.

Semi-solid media have been employed for many years in the study of bacterial motility. Rosenthal (1895) reported marked differences in the size and shape of bacterial colonies which had developed in semi-solid nutrient gelatin. Klie (1896) called attention to the spreading and thread-like appearance of colonies

[1] This work was supported in part by contributions from the General Education Board Research Fund and the Department of Biology, College of Arts and Science, University of Rochester.

of typhoid and colon bacilli in a similar medium. Hiss (1897 and 1902) introduced semi-solid media which were designed to distinguish between typhoid and colon bacilli in either poured plates or stab cultures. Levine (1916) employed semi-solid agar (nutrient broth with 0.5 per cent agar) in culture tubes to determine motility in the *Escherichia-Aerobacter* group. Li (1929) used a semi-solid medium (meat-infusion bouillon plus 0.5 per cent agar and 8 per cent gelatin) in poured plates for the differentiation of motile and non-motile types of the "hog cholera bacillus." Recently, Jordan, Caldwell and Reiter (1934) employed this medium in poured plates to study the motility of *Salmonella Schottmülleri*. Other workers, too, have made use of semi-solid media for the detection of bacterial motility, as evidenced by scattered statements in the literature that hanging-drop results were checked by means of stab cultures in semi-solid media.

During the course of current studies on *Escherichia-Aerobacter* "intermediates," we (1935) obtained perfect correlation between the results of motility determinations made in hanging-drop preparations and in semi-solid agar. Since we knew of no comprehensive investigation comparing the results of the two methods, we undertook such a study. This paper presents the results.

METHODS AND MATERIALS

A total of 1,051 cultures, representing more than 60 species was employed. This number included 665 strains, chiefly members of the *Escherichia-Aerobacter* group, which had been isolated from human urine and feces within 6 months prior to the investigation. The other cultures were selected from the laboratory collection.

The semi-solid medium used in the study was composed of 0.3 per cent meat extract, 0.5 per cent peptone and 0.5 per cent agar. It was adjusted to pH 6.8 to 7.2. It was dispensed in culture tubes and sterilized in the autoclave. Inoculations were made by the stab method with a straight needle. The source cultures were on agar slants or in broth. Incubation was carried

out at 37°C. for 6 days, unless positive results were produced sooner. Hanging-drop preparations were made from either nutrient broth or 2 per cent peptone water cultures, incubated at 37°C. for from 8 to 10 hours; 12 to 15 hours; and 18 to 24 hours.

RESULTS

In the semi-solid agar, motility was manifested macroscopically by a diffuse zone of growth spreading from the line of inoculation. In certain instances, especially with *Aerobacter cloacae* and the motile strains of the *Proteus* group, the growth diffused throughout the medium.

The results of the tests for motility in semi-solid agar and in hanging-drop preparations are recorded in tables 1 and 2. In

TABLE 1

Motility of 1,051 cultures in semi-solid agar and in hanging-drop preparations

MOTILITY	SEMI-SOLID AGAR						HANGING DROPS
	1 day	2 days	3 days	4 days	5 days	6 days	
+	516	538	538	538	538	538	517
−	533	505	496	488	485	481	534
(Nodular)	2	8	17	25	28	32	

hanging-drop preparations, 517 cultures were motile (table 1). In semi-solid agar, after one day, 516 were motile and, after two days, 538 (including all cultures motile in hanging-drop preparations) were motile. Thus, after one day the agar yielded results essentially identical with those of the hanging-drop method; but, after two days, it elicited 4 per cent more positive tests. The only differences between the results of the two methods were found with a single spore-forming culture and certain strains of the *Escherichia-Aerobacter* group, most of which were recently isolated cultures (table 2). A few agar cultures of the *Escherichia* group, after two days, showed evidence of motility by the formation of nodular outgrowths along the stab. Further incubation failed to alter the type of growth in these instances. Isolations from these nodules, and from the non-diffused stab yielded, respectively, motile and non-motile strains

TABLE 2

Motility of various cultures in semi-solid agar and in hanging-drop preparations

ORGANISMS	MOTILITY	SEMI-SOLID AGAR			HANGING DROPS
		1 day	2 days	3-5 days	
Aerobacter	+	24	26	26	25
	−	88	86	86	87
Escherichia "communis"	+	208	213	215	213
	−	188	169	163	185
	(Nodular)	2	6	20	
Escherichia "communior"	+	95	97	98	98
	−	77	73	62	74
	(Nodular)	0	2	12	
Escherichia-Aerobacter "intermediates"	+	78	80	80	80
	−	16	14	14	14
Salmonella	+	46	46	46	46
	−	56	56	56	56
Eberthella	+	11	11	11	11
	−	2	2	2	2
Shigella	+	0	0	0	0
	−	45	45	45	45
Alcaligenes	+	4	4	4	4
	−	0	0	0	0
Proteus	+	35	35	35	35
	−	8	8	8	8
Serratia	+	7	7	7	7
	−	0	0	0	0
Chromobacterium	+	1	1	1	1
	−	0	0	0	0
Pseudomonas	+	6	6	6	6
	−	0	0	0	0
Cocci	+	0	0	0	0
	−	40	40	40	40
Bacillus	+	11	11	11	10
	−	3	3	3	4

identical in other cultural characteristics. Thus, motile strains have been obtained from some cultures which were thought to be non-motile on the basis of hanging-drop determinations.

DISCUSSION

When the results obtained in semi-solid agar are compared with those of the usual hanging-drop technique, it is evident that the production of a diffuse zone of growth around the line of inoculation in the semi-solid agar serves very satisfactorily to indicate bacterial motility. After incubation for one day in semi-solid agar, the results agreed with those of the hanging-drop method for 99.2 per cent of the cultures. Ultimately, every culture which was motile in hanging-drop preparations was motile also in the semi-solid agar. In the case of 22 strains of the *Escherichia-Aerobacter* group, however, it required two days to elicit a positive reaction by the semi-solid agar method. On the other hand, most of the positive reactions were evident after from 8 to 16 hours. The fact that 20 strains of the *Escherichia-Aerobacter* group, and one spore-forming culture were definitely motile in semi-solid agar, but not in hanging-drop preparation, shows that, in certain instances, the former method will indicate motility when the latter does not. The isolation of motile and non-motile strains from nodular outgrowths and from the non-diffused stab, respectively, is considered to be evidence that only a few cells of the parent culture were motile or potentially motile. Thus, the use of stab cultures in semi-solid agar is distinctly advantageous, when only a small proportion of motile cells is present in a culture, for these may be missed entirely either in hanging-drop preparations or when the semi-solid medium is used in poured plates. The advantages of the semi-solid agar method are particularly evident in teaching schedules and routine testing, because the results are cumulative and macroscopic.

SUMMARY

1. A study of semi-solid agar (nutrient broth plus 0.5 per cent agar) for detecting bacterial motility has been made with 1,051

cultures, representing more than 60 species, and the results obtained compared with those of the usual hanging-drop technique.

2. In semi-solid agar, inoculated by the stab method, motility is manifested macroscopically by a diffuse zone of growth which spreads from the line of inoculation.

3. After incubation for one day, the semi-solid agar method gave results essentially identical with those of the hanging-drop, but after two days it showed 4 per cent more positive cultures.

4. The semi-solid agar method permitted the isolation of motile and non-motile strains from some cultures which were non-motile according to the hanging-drop technique.

5. The semi-solid agar method is particularly advantageous in teaching schedules and routine testing, because the results are cumulative and macroscopic.

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A PRELIMINARY NOTE ON THE PREPARATION OF NON-TOXIC SHIGA DYSENTERY VACCINES BY IRRADIATION WITH SOFT X-RAYS

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It is known that intravenous injections of heat-killed suspensions of *Shigella dysenteriae* (Shiga) are so toxic that rabbits are first paralyzed and later succumb with typical symptoms. Non-toxic vaccines may be prepared by treatment with formalin (Costa, Boyer and Placidi, 1925; Durand, 1925; Enlows, 1925; Ramon and Dumas, 1924; Wherry and Bowen, 1925), nitrous acid, (Foshay) or, according to Manoussakis (1932), by permitting the suspensions to remain at ice-box temperatures for five months.

It is also known that bacteria may be killed with x-rays, and that the soft or long wave-length part of the x-ray spectrum is especially lethal. It is possible that the changes accompanying the killing of bacteria with heat are not identical with those produced with x-ray killing and that a vaccine prepared by irradiation of organisms with soft x-rays might be less toxic than one prepared by killing with heat, yet retain all of its antigenic properties. It is the purpose of this paper to describe some experiments which were carried out to test this possibility.

METHODS AND APPARATUS

The bacteria were prepared for irradiation by wiping off eighteen-hour slant cultures of *S. dysenteriae* (Shiga) and bringing the resulting suspensions in salt solution to a turbidity of 500 on the Fuller's scale. In order that the suspension during irradiation would occupy a small enough space to be placed in the most intense part of the x-ray beam near the focal spot of

the tube, it was concentrated by centrifuging for ninety minutes at 4000 r.p.m., decanting the supernatent liquid and resuspending in 1 cc. of sterile saline. These concentrated suspensions were irradiated in small glass dishes placed at a distance of 5 cm. from the focal spot of a copper target gas x-ray tube (Kersten, 1934) whose radiation had the K_α(1.54 Å) and the K_β(1.38 Å) lines of copper as its most intense parts. The tube was operated at 38 peak kilovolts, with a current of 20 m.a. and the time of irradiation varied from 30 to 300 minutes. After irradiation the suspensions were made up to 10 cc. with sterile saline and tested for sterility before use. All the heat-killed suspensions were treated in the same manner, i.e., centrifuged, decanted and resuspended and then heated at a temperature between 55° and 60°C. for one hour. Suspensions of living organisms used as tests for protection were brought to a turbidity of 500 on the Fuller's scale before injection.

During the time of irradiation, the sample, being very near the focal spot of the x-ray tube, was slightly warmed by heat transmitted through the thin window, even though the target and body of the tube were cooled with running water. The approximate temperature of the sample was measured by replacing the suspension of bacteria in the small glass dish, with an equal volume of oil in which was immersed a thermocouple. The replacement of the saline suspension by the oil was necessitated by the fact that the former is an electrical conductor. The temperature was found to rise approximately 3°C. above room temperature under the same conditions of irradiation as that used for the bacterial suspensions.

As a test for the antigenic power of the x-ray vaccines, the animals were given 0.4 cc. of living organisms intravenously. Control animals were given equal doses to determine whether the toxicity of these amounts was sufficient to kill.

RESULTS

Table 1 gives a summary of the results of the inoculation of 45 rabbits with x-ray treated vaccines, and of 12 controls given heat-killed or living organisms. Of 30 animals receiving from

1 to 4 cc. (1 cc. doses) of vaccine irradiated from 30 to 100 minutes, 17 died after receiving one to three inoculations and 13

TABLE 1

Showing the immunizing effects of Shiga dysentery vaccine irradiated with soft x-rays for 30 to 300 minutes

MINUTES OF IRRADIATION	NUMBER OF RABBITS	TOTAL AMOUNT OF VACCINE	DEATH FROM VACCINE	IMMUNITY TO 0.4 CC. LIVING B. DYSENTERIAE (SHIGA) GIVEN INTRAVENOUSLY (DENSITY EQUAL TO VACCINE)
30–60	4	4 cc. (1 cc. every 7 days)	2 (after first inoculation)	2
60	7	4 cc. (1 cc. every 7 days)	0	5 survived. 2 died of pneumonia 10 days after living organisms were given
60	1	3 cc. (1 cc. every 7 days)	1 (after third inoculation)	
60	3	2 cc. (1 cc. every 7 days)	3 (after second inoculation)	
60	10	1 cc.	8 (dead in one to six days	2
70–100	5	4 cc. (1 cc. every 7 days)	3	2
120–300	13	4 cc. (1 cc. every 7 days)	0	13
120–300	1	2 cc. (1 cc. every 7 days)	1 (died after second dose. Gm. + staphylococcus in heart's blood)	
120–300	1	3 cc. (1 cc. every 7 days)	1 (died after third dose. Coccidia in liver. Gm. + coccus in heart's blood)	
Heat-killed vaccine controls	8	1 cc.	8 (died 24 to 36 hours after inoculation)	
Living *S. dysenteriae* (Shiga) suspensions controls	4	0.4 cc.	4 (died 24 to 48 hours after inoculation)	

N.B.: All survivors were watched for three weeks.

survived the test dose of living organisms. Of 15 animals receiving from 1 to 4 cc. (1 cc. doses) of vaccine irradiated for 120

to 300 minutes, 2 died as a result of intercurrent infections and the remainder (13) survived the test dose for immunity. All control animals receiving heat-killed or living organisms died within 24 to 36 hours after inoculation.

From these results it appears that vaccines receiving large doses of x-rays are rendered non-toxic and retain their antigenic properties, while vaccines receiving small doses of x-rays, even though these doses are more than sufficient to merely kill the organism, produce vaccines which are still toxic.

CONCLUSIONS

A non-toxic vaccine of *Shigella dysenteriae* Shiga has been prepared by irradiation of saline suspensions of the organisms with soft x-rays. This vaccine retains its antigenic properties as evidenced by the protection of rabbits against subsequent fatal doses of heat-killed and living suspensions of the organisms.

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AN INTERESTING NEW SPECIES OF LUMINOUS BACTERIA

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The purpose of this paper is to give a full description of an interesting new species of luminous bacteria. Its characteristics make it promising material for investigations dealing with the metabolism of such organisms. At the same time it has been thought desirable to give a more complete account than hitherto recorded of another species, extensively studied in the physiological laboratory of Princeton University (Harvey, 1925; Hill, 1932; Shoup, 1929; Taylor, 1934; and others), and identified as *Bacillus Fischeri* (Beij.) Migula (Migula, 1900; Hill and Shoup, 1929), or more recently, according to newer nomenclature, *Achromobacter Fischeri* (Beij.) Bergey *et al.* (Bergey, 1934; Shoup, 1934; Korr, 1935 a, b; Johnson, 1935, a, b). The cultures of *A. Fischeri*, utilized in the present study were originally isolated by Dr. Korr from a dead squid at Woods Hole, Massachusetts, in the summer of 1933. The new species was isolated by the senior author from a dead amphipod (*Talorchestia*, sp.) at Woods Hole, during the summer of 1935. It is morphologically very similar to *A. Fischeri*, but may be readily distinguished from it, as well as from many other species of luminous bacteria, by virtue of the fact that neither its growth nor luminescence is favored by glycerol, and it produces no acid from this substrate in culture. Other definite physiological and cultural characteristics separate it from all adequately described species that have been reported in the literature.[1] Because of the numerous fundamental con-

[1] Molisch (1912) lists as "Halbwegs gekennzeichnet, teilweise gut beschrieben," 30 species described up to that time. Most of these had been studied by Migula

tributions that have been made by E. N. Harvey, not only with respect to the problem of bio-luminescence in general, but also in regard to the luminous bacteria, the name, *Achromobacter Harveyi*, is proposed for this species.

Although neither of these species was isolated directly from sea water, they are almost unquestionably of marine origin. They thrive best in media made up in natural sea water, and will not grow on ordinary media without the addition of salt, the optimum concentration for which is 2.8 to 3.0 per cent, which approximates that in sea water. Furthermore, in common with most marine organisms, they grow best in alkaline media (a fact noted as early as 1894 by Fischer), and are readily injured by acid. For these reasons the organisms were maintained on the $CaCO_3$-buffered sea water medium of Hill (1928) without the glycerol, since with glycerol in the medium enough acid is produced in a few days by *A. Fischeri* to kill the organisms. In determining acid production from carbohydrates, the medium consisted of 0.2 per cent peptone in sea water, with the addition of 2 cc. saturated aqueous solution of phenol red per liter. This broth was tubed in 9 cc. quantities and sterilized in the autoclave. The carbohydrates, with the exception of starch, inulin, and glycogen, which were sterilized with the medium, were made up in a concentration of 10 per cent (or 5 per cent in the case of a few of the rare sugars) in sea water, sterilized by filtration through a Seitz filter, and added in 1 cc. amounts by sterile pipettes to the tubes of broth. These were incubated to prove sterility prior to inoculation. Inoculations consisted of a small loopful of a 24-hour broth culture, or of a dilute sea water suspension of organisms from a 24-hour agar slant.

It has been observed in these studies that when fixed acid is

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(1900). Giard (1889) reported a species of bacteria causing luminescence of various crustacea, including the amphipod *Orchestia*, but from the rather meager descriptions available, was evidently dealing with a different species. In this connection, compare Lehmann and Neumann (1901). For a comprehensive table giving probable synonymies of most of the species of luminous bacteria that were reported in the earlier literature, together with a table giving a synopsis of their characteristics, see Dahlgren (1915).

produced it appears abundantly and unmistakably, whereas non-fixed acid, such as that from CO_2, is more variable. The fixed or non-fixed nature of the acid was determined seven days after inoculation, by aerating the acid cultures with a stream of air. This brings the CO_2 tension of the medium into equilibrium with the atmosphere, and if the acidity has been brought about by an excess of CO_2, the normal pH of approximately 8.0 is quickly restored. With the exception of *A. Harveyi* with salicin, and *A. Fischeri* with glycerol, the production of fixed acid was always sufficient to cause the pH of the cultures to drop from 8.0 to 6.6 (the acid limit of the indicator) within 24 hours at room temperature, accompanied by a diminution or loss of light production. The controls, without added carbohydrate, never showed any indication of acid production beyond a slight, temporary drop in pH of 0.2 at most, during the period of most rapid multiplication of the organisms.

Gas formation from glucose in fermentation tubes could not be detected with either organism.

ACHROMOBACTER HARVEYI, N. SP.

Rods, 0.5 to 1.0 by 1.2 to 2.5 microns, occurring singly or in pairs, with rounded ends (fig. 10). Rods occasionally slightly curved; ends occasionally slightly pointed. Gram-negative. Spores absent. Capsules absent. Motile by means of a single polar flagellum, 2 to 3 times the length of the cell.

Nutrient sea water agar colonies:[2] Mostly very large, reaching in some cases a diameter of 6 to 8 cm. in 24 hours, flat, highly iridescent, circular with more or less undulant borders, or composed of narrow and close or wide filamentous growth. Occasionally small colonies appear that are circular, with entire or slightly undulate edges, often producing irregular secondary growth (figs. 2 to 6), surface always smooth. Luminescent.

Sea water gelatin, pH 7.0. After 24 hours at 20°C., colonies

[2] In many respects similar to colonies figured by Molisch (1904) for *Microspira photogena* Molisch, a species also related to *A. Fischeri*. *A. Harveyi* is most readily distinguished from *M. photogena* by its action on gelatin, since the latter consistently produced no free liquid.

circular, margin slightly undulate, sunken due to the beginning of liquefaction, about 1.5 mm. in diameter or larger, interior somewhat zonate; colonies surrounded by a "halo" of numerous small secondary colonies, circular and finely granular. As a result of rapid liquefaction, 48-hour colonies lose most of their characteristics. In crowded plates, a large number of gas bubbles present. Luminescent.

Sea water agar slant: growth abundant, spreading, greyish, viscous, homogeneous, iridescent, the medium becoming rapidly alkaline when inoculated at an initial pH of 7. With fish decoctions added to the medium, luminescence is much brighter, and growth becomes brownish after several days.

Growth on autoclaved fish: abundant, smooth, glistening, yellowish, becoming a dirty brown after several days. Mild putrefactive odor. Luminescence very brilliant.

Sea water gelatin stab: rapid saccate liquefaction with much flocculent sediment, the gelatin becoming completely liquefied within 5 days at 22°C. (fig. 8).

Potato plugs resting on cotton saturated with sea water: slight growth, somewhat spreading, slightly brownish, luminous.

Milk, with or without the addition of 2.8 per cent NaCl: no growth.

Sea water containing 0.2 per cent peptone: abundant uniform turbidity, thin pellicle, sediment accumulating over a period of several days. Luminescence at surface only unless the tube is shaken.

Indol produced (tested by Goré's method, 1921).

Hydrogen sulphide produced (tested by method of ZoBell and Feltham, 1934).

Ammonia produced in peptone media (tested by method of Hansen, 1930).

Nitrates reduced.

Starch agar: wide zone of hydrolysis of starch.

Fixed acid in: glucose, fructose, mannose, galactose, sucrose, maltose, mannitol, dextrin, glycogen, trehalose, cellobiose; slowly in salicin.

Non-fixed acid: melizitose; slight acid in sorbitol, disappearing after 24 hours.

No acid in: glycerol, xylose, arabinose, dulcitol, inositol, adonitol, erythritol, arabitol, inulin, lactose, raffinose, rhamnose, fucose, alpha methyl glucoside.

Optimum growth temperature: 35° to 39°C., but grows abundantly also at room temperature of 22° to 25°C.

Optimum temperature for luminescence:[3] 20° to 40°C. Luminescence practically absent at 45°; luminescence reduced at 15°, very weak at 10°, very faint at 5°.

Optimum pH for luminescence: 7.4 to 7.8, weaker at 7.0 and 8.2.

Quality of luminescence[4] to completely dark-adapted eyes: yellowish-green to green on fish, typically green on sea water agar or gelatin, with a tendency to become blue-green on agar slants, especially after several days.

Aerobic, facultative anaerobe (proved by growth in phosphate buffered NaCl peptone broth, completely deaerated by a stream of hydrogen purified over hot platinized asbestos and glass-sealed). After growth has taken place anaerobically luminescence occurs only after a period of 1 to 2 hours' aeration.

Not pathogenic when injected into the peritoneum of white rats. Saprophytic on amphipods, but true pathogenicity for these animals not successfully established.

ACHROMOBACTER FISCHERI (BEIJ.) BERGEY ET AL

Rods, 0.4 to 0.8 by 1 to 2.5 microns, occurring singly or in pairs, with rounded ends. Occasional rods slightly curved, ends slightly pointed (fig. 9). Gram negative. Spores and capsules absent. Motile, but organ of motility not successfully determined.

Nutrient sea water agar colonies: small, circular, smooth,

[3] This was determined by subjecting equivalent portions of the same suspension of organisms to different temperatures. The effect of varying the reaction was determined on equivalent portions of a M/2 NaCl suspension added to M/4 phosphate buffer solutions at different pH values.

[4] In describing a luminous species, the "color of light produced" is of doubtful signficance, since it is highly subjective, and could easily have a different meaning to the eyes of the same individual at different stages of dark-adaptation, and furthermore, may vary with the culture medium, age of the culture, or in general, the intensity of the light.

entire, slightly raised, homogeneous, iridescent. Old colonies become yellowish, with margins slightly serrate.

Sea water gelatin colonies: after 48 hours, colonies small (less than 0.5 mm. in diameter) circular, entire, homogeneous, with slight liquefaction.

Sea water agar slant: growth abundant, greyish to yellowish, smooth, viscous, homogeneous, iridescent. With fish decoctions added to the medium, luminescence is no brighter than on nutrient sea water agar with glycerol, if as bright.

Sea water gelatin stab: slight infundibuliform liquefaction, sometimes slightly beaded, tending to become crateriform in old cultures (fig. 7).

Growth on autoclaved fish: moderate, greyish to yellowish, smooth, glistening, luminescent, no odor of putrefaction.

Potato plugs resting on cotton saturated in sea water: growth fairly abundant, spreading, slightly brownish, luminous.

Milk: no growth. Milk plus 2.8 per cent NaCl: slight growth and luminescence but no action on the milk.

Sea water containing 0.2 per cent peptone: growth moderate, mostly near the surface, very thin pellicle; sediment is found in old tubes.

Indol not produced.

Hydrogen sulphide produced.

Ammonia produced in peptone media.

Nitrates reduced.

Hydrolysis of starch doubtful or very slight.

Fixed acid in: glycerol, glucose, fructose, galactose, mannose, maltose, cellobiose,[5] dextrin, salicin.

Non-fixed acid in: mannitol,[5] melizitose.

No acid in: arabinose, xylose, fucose, rhamnose, sucrose, lactose, trehalose, raffinose, glycogen, inulin, adonitol, dulcitol, inositol, sorbitol, erythritol, arabitol, alpha methyl glycoside.

Optimum growth temperature: 25° to 28°C. No growth at 37°C.

[5] In an earlier study by one of us (Johnson, 1935 a, b) evidence was not obtained, by other methods, for the utilization of these substances by washed cells of *A. Fischeri*.

Optimum temperature for luminescence: 28°C. Weak at 10°, none at 5° or at 37°.

Optimum pH for luminescence:[6] 7.4 to 7.8; less intense at 7.0 and 8.2.

Quality of luminescence: typically greenish.

Not pathogenic to white rats.

Aerobic, facultative.

SUMMARY

A complete description is given of a new species of luminous bacteria, designated *Achromobacter Harveyi*, and morphologically quite similar to *Achromobacter Fischeri* (Beij.) Bergey *et al.* A full account of the latter species is also given, in order to complete and summarize earlier data. *Achromobacter Harveyi* is distinguished from *Achromobacter Fischeri* by its carbohydrate fermentations, much more rapid gelatin liquefaction, indol production, more vigorous growth on agar and in broth, large spreading colonies on agar, and high optimum growth temperature.

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[6] This has been studied by Hill (1928).

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PLATE 1

FIG. 1. Typical colony of *A. Fischeri*, 65 hours at room temperature on Hill's sea water agar medium without glycerol. Scale in millimeters. (The lighter crescent zone on the lower edge was caused by refraction of light by the colony.)

FIGS. 2, 3, 4, AND 5. Colonies of *A. Harveyi*, 42 hours at room temperature, on the same medium.

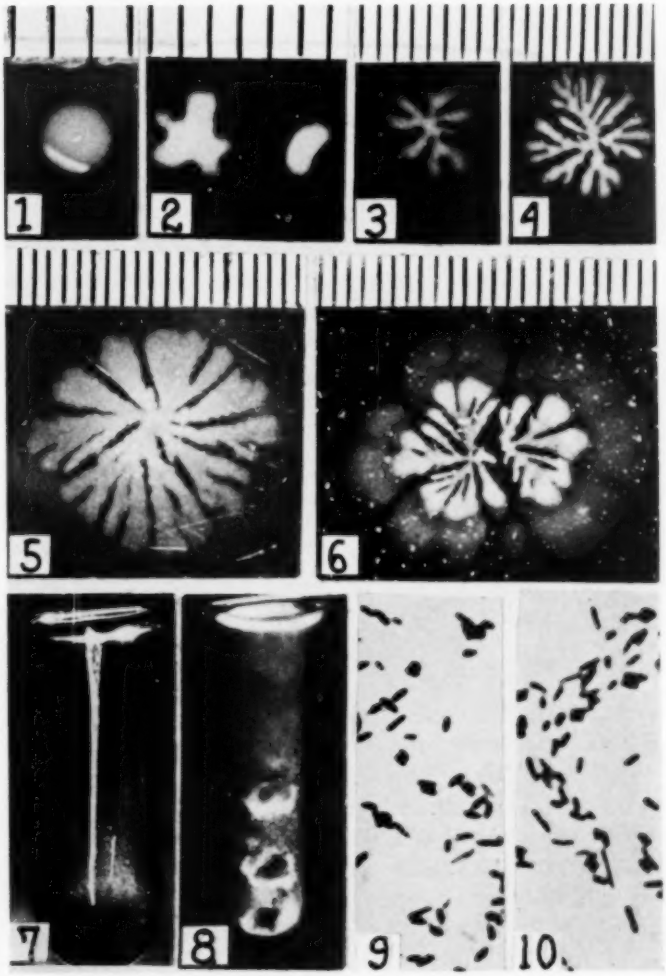
FIG. 6. Colony of *A. Harveyi*, 65 hours old, showing secondary growth. (The small white specks in these photographs are particles of $CaCO_3$ in the medium.)

FIG. 7. *A. Fischeri*, gelatin stab, 4 days at 20° to 22°C.

FIG. 8. *A. Harveyi*, gelatin stab, 4 days at 20° to 22°C.

FIG. 9. *A. Fischeri*, × 2225. From 1-day-old agar slants, stained with crystal violet.

FIG. 10. *A. Harveyi*, × 2225. From 1-day-old agar slants, stained with crystal violet.



(Frank H. Johnson and I. V. Shunk: New Species of Luminous Bacteria)

THE AEROBIC DISSIMILATION OF LACTIC ACID BY THE PROPIONIC ACID BACTERIA[1]

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Fitz in 1878 suggested that the anaerobic dissimilation of lactic acid by the propionic acid bacteria occurs in accordance with the reaction of equation 1. The formation of the end products in substantially the proportion shown has been frequently confirmed.

(1) $$3CH_3 \cdot CHOH \cdot COOH \rightarrow 2CH_3 \cdot CH_2 \cdot COOH + CH_3COOH + CO_2 + H_2O$$

Van Niel (1928) suggested that pyruvic acid is an intermediary which is dissimilated according to equation 2.

(2) $$CH_3 \cdot CO \cdot COOH + H_2O \rightarrow CH_3 \cdot C \cdot (OH)_2 \cdot COOH \rightarrow CH_3COOH + CO_2 + 2H$$

The hydrogen reacts with lactic acid to form propionic acid. Pyruvic acid has been isolated from the fermentation of glucose (1934) and of lactic acid (unpublished data) by Wood and Werkman. These investigators supported Van Niel's proposed dissimilation of pyruvic acid inasmuch as the occurrence of acetaldehyde could not be demonstrated. Little information is available concerning the aerobic dissimilation of lactic acid. Virtanen (1923) found that aeration had slight influence on the fermentation while Van Niel reported a considerable increase in acetic acid when he employed vigorous aeration.

As part of a general study of the respiration of the propionic

[1] Supported in part by Industrial Science Research Funds of Iowa State College.

acid bacteria the present investigation was conducted on the aerobic dissimilation of lactic acid. The experimental technic is unusual in that both the oxygen consumed and the final products have been determined. The bearing of the results on the mechanism of the propionic dissimilation is discussed.

METHODS

Micro-manometric technic using Barcroft-Warburg constant-volume manometers was used in preliminary investigations (Dixon, 1934). The bacteria were grown at 30°C. for 3 to 6 days in a medium of yeast extract (Difco) 0.4 per cent and glucose 1.0 per cent. They were washed three times in saline solution. One milliliter of bacterial paste was added to 9 ml. of saline and kept at 6°C. overnight before use. Two milliliters of a mixture of 12 ml. of 0.15 M phosphate buffer, 2 ml. of 1.6 per cent lactic acid, and 2 ml. of bacterial suspension were used in the reaction flasks. The solutions were autoclaved at 15 pounds for 5 minutes and the manometer flasks sterilized with chromic acid. The temperature of the bath was 30°C. The flasks were oscillated continuously 100 times per minute through a 5 cm. amplitude.

The macro-respirometer method as described by Wood, Erb and Werkman (1936) was used to make a complete quantitative study of the dissimilation. In order to obtain a large yield of organisms $NaHCO_3$ (0.5 per cent) was used as buffer. The bacterial mass obtained by centrifuging was either suspended in phosphate, aerated 12 hours, and then recentrifuged or the mass was used without washing or further treatment. Five grams of the bacterial paste were suspended in 500 ml. of medium consisting of 0.15 M potassium phosphate buffer at pH 5.6 and 0.7 per cent lactic acid neutralized with NaOH solution to the same pH. Incubation was at 30°C. and for 31.5 hours in experiment 1, and for 68.5 hours in experiments 2 and 3.

The lactic acid was determined by the Friedemann and Kendall (1929) method after clarifying with phosphotungstic acid. Pyruvic acid was determined by the iodoform method (Wendel, 1932), after the bacteria were removed by precipitation with 5

per cent trichloracetic acid. Volatile acids were determined by the partition method of Osburn, Wood and Werkman (1933). The steam distillate was concentrated and redistilled to eliminate pyruvic acid from the volatile acid fraction. CO_2 was determined by titration or direct measurement as described by Wood and Werkman (1935). Purity of all cultures was verified at the conclusion of the experiment.

TABLE 1

Influence of pH on the formation of carbon dioxide and utilization of oxygen in the aerobic dissimilation of lactic acid

Time: 8 hours

INITIAL pH	ENDOGENOUS		LACTIC ACID		NET TOTALS		FINAL pH	RATIO $CO_2:O_2$
	O_2	CO_2	O_2	CO_2	O_2	CO_2		
			Part 1					
			By *Propionibacterium arabinosum* (34W)*					
	mm.³	mm.³	mm.³	mm.³	mm.³	mm.³		
5.35	255.5	226.3	876.8	833.6	621.3	607.3	5.6	0.98
5.65	256.0	223.5	743.8	644.0	487.8	420.5	6.0	0.86
6.20	310.5	186.9	807.9	523.3	497.4	336.4	6.8	0.68
7.10	281.1	218.6	687.9	384.6	406.8	166.0	6.6	0.40
			Part 2					
			By *Propionibacterium pentosaceum* (49W)*					
5.45	225.4	204.9	662.6	502.3	437.2	297.4	5.85	0.68
5.75	229.4	208.4	597.8	334.9	368.4	126.5	6.00	0.35
6.25	224.4	179.3	592.0	303.5	367.6	124.2	6.50	0.34
7.00	231.9	188.8	570.4	338.6	338.5	149.8	6.90	0.43

* Age of cells = 72 hours + 13 hours in icebox.

EXPERIMENTAL

The influence of change in hydrogen ion concentration on respiration was first determined (table 1). The oxygen consumed and the CO_2 produced per hour are shown in table 2. The net values were calculated by substracting the gas volumes observed with no substrate from those in which the substrate was included. Ratios are calculated on the basis of net values.

The results using the macro-respirometer and with complete

determination of the products are shown in table 3. Table 4 shows the rates of dissimilation of lactic acid and corresponding formation of products.

TABLE 2

Production of carbon dioxide and oxygen utilized in the aerobic dissimilation of lactic acid

TIME	ENDOGENOUS		LACTIC ACID		NET RATE PER HOUR		NET TOTALS		RATIO $CO_2:O_2$
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	
Part 1. Initial pH 5.65; final pH 6.00									
By *Propionibacterium arabinosum* (34W)*									
hours	mm.³	mm.³	mm.³	mm.³	mm.³	mm.³	mm.³	mm.³	
1	74.7	52.0	307.0	88.0	232.3	36.0	232.3	36.0	0.16
2	54.6	41.1	86.9	102.6	32.3	61.5	264.6	97.5	0.37
3	32.5	34.9	80.5	94.2	48.0	59.3	312.6	156.8	0.50
4	20.1	20.9	63.7	81.6	43.6	60.7	356.2	217.5	0.61
5	22.1	19.4	63.7	76.2	41.6	56.8	397.8	274.3	0.69
6	15.6	17.7	49.8	72.4	34.2	54.7	432.0	329.0	0.76
7	19.5	19.6	48.1	65.0	28.6	45.4	460.6	374.4	0.81
8	16.9	17.9	44.0	64.0	27.1	46.1	487.7	420.5	0.86
Part 2. Initial pH 5.75; final pH 6.00									
By *Propionibacterium pentosaceum* (49W)*									
1	52.6	36.4	136.1	27.5	83.5	−8.9	83.5	−8.9	
2	42.9	34.1	122.2	35.6	79.3	1.5	162.8	−7.4	
3	33.1	30.0	118.8	35.3	85.6	5.3	248.4	−2.1	
4	27.3	28.4	68.9	48.6	41.6	20.2	290.0	18.1	0.06
5	20.1	21.6	39.4	18.5	19.3	−3.1	309.3	15.0	0.05
6	20.8	22.3	38.8	34.0	18.0	11.7	327.3	26.7	0.08
7	16.9	18.5	37.1	33.3	20.2	14.8	347.5	41.5	0.12
8	15.6	17.0	36.5	19.4	20.9	2.4	368.4	43.9	0.12

* Age of cells = 72 hours + 13 hours in icebox.

In this experiment (table 1) the lowest pH (5.35) gave the largest oxygen uptake and CO_2 production. Data not shown indicate that the dissimilation falls off abruptly at lower pH values and also that the optimal pH for the production of CO_2 from lactic acid under anaerobic conditions is in the same range. These values are quite different from those for optimal growth

which has been found to be near pH 7.0. It is of interest that the ratios of CO_2:O_2 show such a regular variation. Increase in pH resulted in a decrease in the ratio. The absorption of

TABLE 3

Aerobic dissimilation of lactic acid by Propionibacterium arabinosum (culture 84W)

NUMBER	LACTIC ACID FERMENTED PER LITER	PRODUCTS PER 100 mM OF FERMENTED LACTIC ACID					O_2 UTILIZED PER 100 mM OF FERMENTED LACTIC ACID	CARBON RECOVERED	REDOX INDEX*
		Total volatile acid	Propionic acid	Acetic acid	CO_2	Pyruvic acid			
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*per cent*	
1	81.3	35.0	2.7	32.2	35.2	67.0	64.4	103.0	1.04
2	80.3	87.0	14.0	73.0	61.0	20.4	62.1	103.4	1.03
3	79.5	103.5	27.4	76.1	77.9	0.0	67.6	104.1	0.96

* Calculated as follows: each molecule of hydrogen (H_2) has a reduction value of 1, each atom of oxygen (O) has an oxidation value of 1, therefore H_2O = O, CO_2 = oxidation value of 2, CH_3CH_2COOH = reduction value of 1. (Cf. Johnson, Peterson and Fred, 1931). Redox index = Ratio of sum of products of oxidation value of each product x millimoles to sum of products of reduction value of each product x millimoles. Perfect redox index = 1 since oxidation is accompanied by equivalent reduction.

TABLE 4

Aerobic dissimilation of lactic acid

TIME	LACTIC ACID FERMENTED	PYRUVIC ACID	CO_2	O_2	CO_2:O_2
hours	*mM*	*mM*	*mM*	*mM*	
2	14.8	10.8	0.8	5.4	0.15
4	28.8	19.8	2.7	11.5	0.23
10	50.8	37.2	11.2	27.6	0.41
14.3	63.1	47.2	15.5	36.1	0.43
22	76.9	54.3	25.0	48.5	0.52
28.8	80.4	55.3	28.3	52.0	0.54
31.2	81.3	54.4	28.6	52.3	0.55

Results calculated on the basis of 1 liter of medium.

CO_2 by the buffer may have had some influence but at the lower pH values the factor is negligible. It is apparent that the mechanism of dissimilation is influenced by pH.

The ratios of CO_2:O_2 shown in table 2 are significant. In

the experiment tabulated in part 1 the ratios are less than 1 and increase with time; in part 2 the ratios are practically 0 throughout the dissimilation. The uptake of oxygen unaccompanied by an equivalent production of CO_2 suggests the formation of an oxidized 3-carbon compound. The propionic acid bacteria contain an enzyme capable of catalyzing the following conversion:

(3) $$CH_3 \cdot CHOH \cdot COOH \rightarrow CH_3 \cdot CO \cdot COOH + 2H$$

The introduction of the enzyme (bacterial suspension) into a solution of lactic acid would lead to the establishment of the equilibrium by formation of pyruvic acid. If oxygen acts as the hydrogen acceptor the ratio $CO_2:O_2$ would be 0 increasing to 1 upon oxidation of the pyruvic acid to CO_2 and acetic acid. In experiment 2, part 1, 47.6 per cent of the oxygen consumed was utilized in the first hour suggesting a rapid formation of pyruvic acid. Following this the CO_2 production and the ratio of $CO_2:O_2$ increased indicating that the accumulated pyruvic acid may have been dissimilated to acetic acid and CO_2. The final value of the ratio approaches that for the oxidation of lactic acid to acetic acid and CO_2. In part 2, it is probable that pyruvic acid accumulated without significant destruction.

It is evident that the above suggestions based only on a knowledge of the gaseous products are in need of confirmation. For this reason the quantitative determination of all products was made using the macro-respirometer. Results are given in table 3. It was proven that pyruvic acid is a product of dissimilation. The acid was identified by preparation of the 2,4-dinitrophenylhydrazone. Melting point, 213°C.; m.p. of authentic preparation 213°C.; mixed m.p. 213°C. With these new facts it is evident that the function of pyruvic acid as an intermediate in the propionic acid fermentation may be considered well established, for the acid has been isolated by two independent methods (sulfite fixation (Wood and Werkman, 1934) and aerobic dissimilation).

The data in table 3 are of interest from another point of view. The authors have not found in the literature an investigation of

an aerobic dissimilation in which the substrate fermented, products formed and oxygen utilized were quantitatively determined, permitting calculation of the carbon balance and redox index under aerobic conditions. In the calculation of the redox index it was assumed that the oxygen is reduced to water. The balances confirm this assumption and hydrogen peroxide was not detected.

Aerobic experiments often have been unsatisfactory when compared to anaerobic for they have lacked the completeness of the latter. In anaerobic experiments, since hydrogen donators or acceptors other than those of the substrate are not involved, there are formed equivalent quantities of oxidized and reduced compounds. In aerobic experiments the functioning of oxygen as a hydrogen acceptor complicates calculations. In the present experiments we have overcome this difficulty by determining the oxygen utilized. One can thus account quantitatively for the effect of the oxygen and calculate oxidation-reduction balances.

The mechanism of the aerobic fermentation of lactic acid appears to be substantially the same as in anaerobic dissimilation with the exception that oxygen acts as the hydrogen acceptor thus causing a decreased production of propionic acid. The acetic acid and CO_2 occur in practically equal molar quantities in 2 cases (table 3) indicating that the pyruvic acid is oxidized to acetic acid and CO_2 and that there is no oxidation of the propionic and acetic acids to CO_2. In experiment 2 the CO_2 is not equivalent to the acetic acid. Possibly in this case some CO_2 was utilized synthetically as observed in glycerol fermentation (Wood and Werkman, 1936).

The factors governing the formation of pyruvic acid have not been thoroughly investigated. Apparently the treatment afforded the bacteria is important. Cells which were centrifuged, suspended in phosphate, aerated for 12 hours and then recentrifuged produced pyruvic acid (experiments 1 and 2) while those used directly after centrifuging did not show any accumulation of this acid (experiment 3). This effect may be due to the washing of the bacterial cells rather than to the aeration, for the results shown in table 2 were obtained with washed but unaerated

cells. The fact that treatment of the cells is related to pyruvic acid destruction is shown also by our investigations with pyruvic acid in which it was found that aerated cells destroy pyruvic acid very slowly while with non-aerated cells the attack is vigorous.

The data in table 4 are from experiment 1, table 3. In this experiment the CO_2 was determined by measuring the volume of gas. The same type of change in the ratio of CO_2:O_2 occurred as in experiments using the micro-respirometer. The lactic acid fermented and pyruvic acid formed were also determined. At the conclusion there was a large quantity of pyruvic acid present but its breakdown was slight as shown by the small increase in the quantity of CO_2 in the latter part of the experiment.

SUMMARY AND CONCLUSIONS

Experimental results obtained by gas measurements show that the propionic acid bacteria utilize oxygen in their dissimilation of lactic acid. The most favorable pH for oxygen uptake was found to be 5.3 to 5.6. Pyruvic acid was shown to be a product of the dissimilation under the conditions of our experiments. Products of the dissimilation of lactic acid and the oxygen utilized were quantitatively determined and carbon and oxidation-reduction balances calculated thus offering more complete information on the mechanism of aerobic dissimilation of lactic acid by propionic acid bacteria.

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DIVERSION OF THE NORMAL HETEROLACTIC DISSIMILATION BY ADDITION OF HYDROGEN ACCEPTORS[1]

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A clear understanding of the dissimilation of carbohydrates by the heterofermentative lactic acid bacteria would be of assistance in problems of classification of the group and should prove of especial value to the dairy industry in the study of flavors and aromas. Our knowledge of the dissimilation of the heterolactic bacteria is extremely fragmentary at present. Peterson and Fred (1920) working with *Lactobacillus pentoaceticus* suggested the breakdown of glucose to ethyl alcohol, CO_2 and lactic acid with a secondary fermentation of the lactic to acetic acid and CO_2. These authors have found evidence for the occurrence of acetaldehyde as an intermediary. Nelson and Werkman (1935) have studied the fermentation of pyruvic acid by *Lactobacillus lycopersici* and found one molecule to be oxidized to acetic acid and CO_2 with a simultaneous reduction of a second molecule to lactic acid. The same authors (1935a) have proposed a scheme of dissimilation of glucose by the heterofermentative lactic bacteria in which pyruvic acid and acetaldehyde are intermediaries. An understanding of the behavior of added hydrogen acceptors would be of considerable value in formulating schemes of dissimilation or in determining the probability of schemes already proposed.

In the present investigation suitable hydrogen acceptors (acetaldehyde and acetylmethylcarbinol) were added to fermentations of glucose by *Lactobacillus lycopersici*, a typical heterolactic species, and the influence of the reduction of the acceptor upon the

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normal course of dissimilation determined. This method of attack permits certain conclusions as to the mechanism of the normal dissimilation, particularly as to the behavior of intermediary acetaldehyde and as to the source of hydrogen taking part in the transference to acceptors.

METHODS

Lactobacillus lycopersici was described by Pederson (1929) to whom we are indebted for the culture used in this investigation.

The medium consisted of glucose, 2 per cent; yeast extract (Difco), 0.2 per cent; peptone, 1 per cent; K_2HPO_4, 0.6 per cent; and KH_2PO_4, 0.6 per cent. Solutions of (1) glucose, (2) yeast extract and peptone and (3) phosphates were sterilized separately at 20 pounds for 20 minutes and combined just prior to inoculation. One liter of medium was placed in each 1500 cc. Erlenmeyer flask, adjusted to pH 6.2 and inoculated with 25 cc. of a 3-day culture. The acetaldehyde was sterilized by Seitz filtration. Quantities of 0.36 and 0.72 gram of aldehyde were added to experiments II and III respectively on the third, sixth and tenth days of fermentation. The acetylmethylcarbinol was sterilized in the same manner and added at the time of inoculation.

The acetaldehyde volatilizing during fermentation was caught as it passed along with nitrogen, bubbled through the medium, into a bead tower containing a 10-per-cent solution of sodium bisulfite. It was determined by iodine titration. Since sulfur dioxide is liberated from the sodium bisulfite standing at 30°C. for the time required for completion of the fermentation, it was necessary to place a potassium permanganate solution between the bisulfite tower and the Bowen potash bulbs to prevent interference with the carbon dioxide determination. Unfermented acetaldehyde was distilled into a sodium bisulfite solution and determined by iodine titration.

The carbon dioxide formed during the fermentation was carried into Bowen potash bulbs by a stream of oxygen-free nitrogen (bubbled intermittently through the medium) and determined gravimetrically. The residual carbon dioxide was determined by refluxing an aliquot part of the medium with a stream of carbon-

dioxide-free air passing through to carry the carbon dioxide into Bowen potash bulbs for gravimetric analysis.

The volatile acids were determined in 400 cc. of medium by the method of Osburn, Wood and Werkman (1933). Succinic acid was determined by preparation of the silver salt and lactic acid by the method of Friedemann, Cotonio and Shaffer (1927).

Ethyl alcohol was determined by oxidizing the alcohol to acetic acid. Before oxidation, however, it was necessary to remove the unfermented acetaldehyde by precipitation with 2,4-dinitrophenylhydrazine. The precipitate was removed by filtration, the filtrate neutralized and the alcohol distilled off and determined by the method of Stahly, Osburn and Werkman (1934).

Glycerol was extracted with ethyl ether and determined by the method of Wagenaar (1911).

Sugar was determined at the time of inoculation and at the conclusion of the fermentation by the Munson and Walker (1906) method after deproteinating with phosphotungstic acid and removing the acetaldehyde by distillation. Acetylmethylcarbinol and 2,3-butyleneglycol were determined by the methods of Van Niel (1927) and Brockmann and Werkman (1933), respectively, as modified by Stahly and Werkman (1936). Corrections for the effect of acetylmethylcarbinol on the determinations of glucose, ethyl alcohol and 2,3-butyleneglycol were made.

Purity of the cultures was determined by microscopic and cultural examination at the time of inoculation and just prior to analysis.

EXPERIMENTAL

A typical experiment showing the effect of the addition of acetylmethylcarbinol to a glucose fermentation is given in table 1. The carbinol was reduced completely to 2,3 butylene-glycol, resulting in a decrease in the quantities of ethyl alcohol, lactic acid and glycerol with a corresponding increase in acetic acid and carbon dioxide.

The increase in acetic acid was 37.4 mM while the increase in carbon dioxide was only 19.1 mM, but since the ethyl alcohol was decreased by 19.3 mM and each molecule of alcohol is accom-

panied by a molecule of carbon dioxide the sum of the two (38.4 mM) equals the CO_2 to accompany the increase in acetic acid (cf. fig. 1). The decrease in ethyl alcohol plus the decrease in lactic acid (40.7 mM) can be accounted for by the increase in acetic acid.

Since lactic acid is fermented aerobically to acetic acid and carbon dioxide, the added acetylmethylcarbinol may replace oxygen and bring about the breakdown of the lactic acid anaerobi-

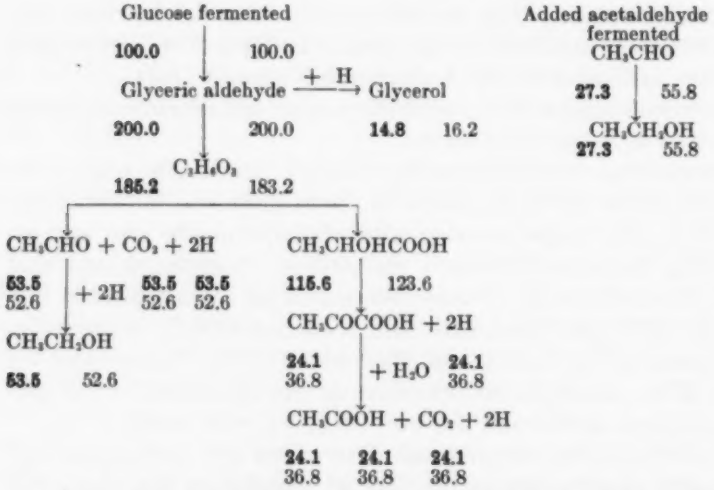

FIG. 1. THE EFFECT OF ADDED ACETALDEHYDE ON THE DISSIMILATION OF GLUCOSE BY L. LYCOPERSICI

The figures in bold-face are the data of experiment II; others, of experiment III (Table 2).

cally. However, a decrease in ethyl alcohol and glycerol must also be accounted for. This may be done by postulating a reduction of acetylmethylcarbinol by acetaldehyde. The latter is dehydrogenated to acetic acid and not reduced to ethyl alcohol. The carbinol is reduced by two sources of hydrogen: (1) by that which normally reduces the aldehyde and (2) by the hydrogen liberated by the dehydrogenation of the acetaldehyde which normally would be reduced.

Inasmuch as both ethyl alcohol and lactic acid are diminished, it is probable that the acetylmethylcarbinol reacts with a common precursor of lactic acid and ethyl alcohol. Pyruvic acid is the precursor of both in the Embden-Meyerhof scheme of muscle or yeast glucolysis (Embden, Deuticke and Kraft 1933; Meyerhof, 1933). The isolation of phosphoglyceric acid in the dissimilation of glucose by lactic bacteria (Stone and Werkman, unpublished data) supports the occurrence of the initial stages of the Embden-Meyerhof scheme in bacterial dissimilation. In this case pyruvic

TABLE 1

The effect of the addition of acetylmethylcarbinol on the dissimilation of glucose by L. lycopersici

	I	II	DIFFERENCE (II − I)
	mM	*mM*	*mM*
Glucose fermented	102.2	101.7	−0.5
Acetylmethylcarbinol fermented	0	54.1	+54.1
2,3-Butyleneglycol	0	60.5	+60.5
Ethyl alcohol	74.1	54.8	−19.3
Acetic acid	15.5	52.9	+37.4
Carbon dioxide	81.0	100.1	+19.1
Lactic acid	83.1	61.7	−21.4
Glycerol	32.6	25.3	−7.3
Percentage of C recovered	99.0	97.1	
Oxidation-reduction index	0.88	0.97	

acid will be formed from phosphoglyceric acid and reduced to lactic acid. Ethyl alcohol will be formed by the decarboxylation of pyruvic acid to acetaldehyde which is in turn reduced to ethyl alcohol. If pyruvic acid is dehydrogenated to acetic acid and CO_2 by acetylmethylcarbinol, a corresponding sparing of the hydrogen donators (α-glycerolphosphoric acid?) occurs, one mole for each mole of acetic acid formed.

In the fermentation of glucose to which acetaldehyde is added as a hydrogen acceptor, diversion of the course of dissimilation is

more complex owing to the occurrence of ethyl alcohol as a normal product. Typical experiments in which acetaldehyde is added are shown in table 2. Diminution of ethyl alcohol formed from glucose is not apparent for the reason stated but in all probability occurs; acetic acid is increased, and glycerol and lactic acid decreased in agreement with the behavior of acetylmethylcarbinol. CO_2 shows a probable increase; it is likely that the determination 71.5 mM (table 2, II) is somewhat low as suggested by the low carbon recovery of 92.6 per cent. The results are similar to those

TABLE 2

The effect of the addition of acetaldehyde on the dissimilation of glucose by L. lycopersici

	I	II	III	DIFFERENCE (III − II)
	mM	*mM*	*mM*	*mM*
Glucose fermented	100	100	100	
Acetaldehyde fermented	0	27.3	55.8	+28.5
Ethyl alcohol	59.3	80.8	108.4	+27.6
Acetic acid	18.2	24.1	36.8	+12.7
Lactic acid	94.1	91.4	86.8	−4.6
Glycerol	30.4	14.8	16.2	+1.4
Carbon dioxide	74.6	71.5	79.5	+8.0
Percentage of carbon recovered	102.0	92.6	95.5	
Oxidation-reduction index	1.00	0.96	0.90	

with acetylmethylcarbinol. Differences between II and III, table 2, may be interpreted in the same manner as I and II, table 1. The differences in acetaldehyde or acetylmethylcarbinol fermented are equivalent to the ethyl alcohol or 2,3-butyleneglycol formed, indicating reduction of the added acceptor. Assuming complete reduction of the 27.3 mM acetaldehyde, an equivalent quantity of hydrogen is diverted from the normal dissimilation and in addition 5.9 mM of hydrogen will be provided owing to the increased dehydrogenation of acetaldehyde to acetic acid, leaving a decrease of 21.5 mM H_2 available for reduction. The calculated decrease amounts to 24.1 mM, a satisfactory agree-

ment in view of the analytical difficulties and low carbon recoveries. Exactly identical claims can not, of course, be made for acetylmethylcarbinol and acetaldehyde, since the latter is competing with biologically formed acetaldehyde whereas acetylmethylcarbinol may be a better acceptor of hydrogen.

An application of the data in table 2 to the outline of Nelson and Werkman (1935a) is shown in figure 1.

The sum of values found for acetic acid, lactic acid and ethyl alcohol for experiment II is 169 (196.3–27.3) mM while the theoretical value is 185.2 mM; for experiment III the values are 176.3 mM and 183.2 mM respectively.

The quantity of active hydrogen formed by the dissimilation of lactic acid should be equivalent to the quantity of ethyl alcohol formed from the acetaldehyde added plus the millimoles of glycerol formed, since a sufficient quantity of active hydrogen to reduce the aldehyde formed from the glucose is produced in the breakdown of the 3-C compound to acetaldehyde, CO_2 and active hydrogen. The calculations made from the data show for experiment II the mM of active hydrogen formed to be 48.2. The quantity of active hydrogen accepted by added acetaldehyde is 27.3 mM; by glyceric aldehyde 14.8 mM making a total of 42.1 of the 48.2 mM of active hydrogen formed accepted. In experiment III the total quantity of active hydrogen formed is 73.6 mM; 55.8 mM are accepted by the added acetaldehyde and 16.2 by the glyceric aldehyde making a total of 72.0 mM accounted for. These values assume that the acetaldehyde is transformed quantitatively to ethyl alcohol and the agreement obtained lends support to this assumption.

In view of the evidence that the added acetaldehyde is reduced to ethyl alcohol, it is probable that the ethyl alcohol formed from glucose by the heterofermentative lactic acid bacteria arises from the reduction of intermediately-formed acetaldehyde as suggested by Nelson and Werkman (1935a).

Added hydrogen acceptors will compete with the acceptors arising from the dissimilation. It is logical to assume that these compounds which are decreased are formed by the reduction of intermediary hydrogen acceptors. The decrease of lactic acid

is in agreement with expectations of the Embden-Meyerhof mechanism of dissimilation. Pyruvic acid acts as a hydrogen acceptor to form lactic acid. Occurrence of this reaction has been shown by Nelson and Werkman (1935) in the case of *Lactobacillus lycopersici*.

SUMMARY

Acetaldehyde and acetylmethylcarbinol added to fermentations of glucose by *Lactobacillus lycopersici* are readily hydrogenated.

Added acetylmethylcarbinol results in an increase in the quantities of acetic acid, and carbon dioxide and a decrease in the quantities of ethyl alcohol, lactic acid and glycerol formed from glucose. Added acetaldehyde functions in a similar manner. The increase in ethyl alcohol is due to hydrogenation of the added aldehyde.

It is suggested that the mechanism of heterolactic dissimilation may involve the essentials of the Embden-Meyerhof scheme. Further discussion of the application of the Embden-Meyerhof scheme to the lactic fermentation will be made.

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STUDIES ON HEMOLYTIC STREPTOCOCCI

II. STREPTOCOCCUS PYOGENES

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NOMENCLATURE

In 1884 Rosenbach described the streptococcus of wound infections and gave it the specific name *Streptococcus pyogenes*. Later, Klein described a streptococcus which he found associated with scarlet fever, and gave it the specific name *Micrococcus scarlatinae*. Arloing found a streptococcus associated with puerperal fever, and named it *Streptococcus puerperalis*, although in the same lecture in which he gave that name he contended that the streptococcus of puerperal fever is the same as that which may cause erysipelas or wound infections.

Prior to Rosenbach's description of *S. pyogenes*, Fehleisen had demonstrated that a streptococcus is associated with erysipelas. Rosenbach compared Fehleisen's streptococcus of erysipelas with the streptococcus of wounds, and found them different. He gave the name *S. erysipelatis* to Fehleisen's streptococcus. On account of the technical limitations of the early bacteriologists, Rosenbach was unable to describe the streptococci of wounds and of erysipelas fully enough to permit their identification at the present time. Nevertheless it is reasonable to accept his statement that he found differences between the streptococci from the two sources.

The name *S. erysipelatis* was adopted by Birkhaug for the streptococci which he found associated with erysipelas. The writer has studied some of Birkhaug's strains, and found that a few of them fall into a well defined group clearly distinguishable from other hemolytic streptococci. These peculiar Birkhaug

strains have been distributed widely to many laboratories in this and other countries under the name *S. erysipelatis*. Hence it is logical to retain that name for the small distinct group. The description of *S. erysipelatis* will be given in a later paper of this series. The present preliminary discussion was occasioned by the fact that some writers who have considered the nomenclature of streptococci have found it necessary to offer excuses for not recognizing the priority of the specific name *erysipelatis* for the main group of hemolytic streptococci. No such excuse is necessary if the name is applied to another distinct species of streptococcus capable of causing erysipelas.

There has never been described any character which would distinguish the three alleged species *pyogenes*, *scarlatinae* and *puerperalis*. It has been claimed that they possess a disease specificity correlated with agglutinative types. Recent investigators, however, (Williams; Griffith (1935)) are agreed that streptococci from scarlet fever or puerperal fever or erysipelas may fall into the same serological group with streptococci from other disease sources; and that scarlet fever, puerperal fever or erysipelas may be caused by more than one agglutinative type of streptococcus. Hence the system of classification of streptococci based on disease specificity is invalid, and must be replaced by a new system.

Andrewes and Christie arrived at the conclusion that *S. pyogenes* (Rosenbach) is the name that should be adopted for the large group of hemolytic streptococci to which the names *pyogenes*, *scarlatinae* and *puerperalis* have been given. Griffith (1935) accepted the name *S. pyogenes* for those streptococci which produce a zone of hemolysis when planted on the surface of horse blood agar, conform to certain colonial appearances, and are associated with outbreaks of disease in man.

The writer accepts the name *S. pyogenes* for the large group of hemolytic streptococci which includes the majority of strains from scarlet fever and from erysipelas, and also includes almost half of the puerperal fever strains in our collection. It includes a greater percentage of strains from acute suppurative diseases than any other group. The specific characters as defined by

Griffith are not distinctive, however. It was pointed out in the first paper of this series that on account of the variation within a given species, colony appearance is not of much use for classification purposes. Griffith's proposal that the name *S. pyogenes* be limited to hemolytic streptococci pathogenic for man does not offer a distinctive character, because some hemolytic streptococci pathogenic for man differ so widely from *S. pyogenes* that they obviously belong to other species; and on the other hand *S. pyogenes* may attack lower animals as well as man. The latter point will be discussed further on.

It is the purpose of this paper to describe those characters of *S. pyogenes* which differentiate it from other species of streptococci which cause clear hemolysis on blood agar plates.

CHARACTERS OF STREPTOCOCCUS PYOGENES

The differential characters[1] of *S. pyogenes* useful for routine identification purposes are as follows: Resistance to nascent phage D/693; resistance to lytic filtrate B/563; sensitivity to phages B/563, C/594 and C/646 in the nascent state. (There are quantitative differences in the sensitivity of the various strains to phage C, some being more readily lysed than others, but all *S. pyogenes* strains are at least slightly sensitive to both C/594 and C/646.) Lactose, salicin and trehalose are fermented; sorbitol and mannitol are not fermented.

Other characters are as follows: final hydrogen ion concentration in glucose broth is pH 5.0 or higher; sodium hippurate is not hydrolyzed; a few strains are capable of growth on 10 per cent bile agar, but none grew on 40 per cent bile agar. The optimum temperature is 37°C. Growth occurs more slowly at 20°C.

Of 33 strains of *S. pyogenes* tested, all but one were found to be capable of dissolving human fibrin. There are marked quantitative differences between the strains, as evidenced by the time required for lysis. The data are given in table 1. Tillett reported similar differences in the fibrinolytic activity of the human strains which he tested.

[1] The methods for determining the characters were described in the first paper of this series.

Virulence of *S. pyogenes* for mice is generally low, as compared with the majority of animal strains of hemolytic streptococci. Of 21 strains tested, 12 failed to kill mice in doses of 0.01 cc. One exceptional strain killed in a dose of 0.00000001 cc. in the third mouse of a series of rapid passages. There was every degree of virulence between the two extremes. (Streptococci of certain other species which are regarded as highly virulent for mice, kill regularly in doses of 0.00000001 cc.)

Three strains of *S. pyogenes* from cases of erysipelas were tested for ability to produce erysipelas in rabbits. One-tenth cubic centimeter of 24-hour blood broth culture was injected intradermally into the tip of the shaved ear. One culture produced no effect. One produced a rise in temperature to 40°C. on the

TABLE 1

Time required for lysis of human fibrin by S. pyogenes

TOTAL NUMBER OF CULTURES TESTED	++++ INHIBITION OF CLOTTING		+++ DISSOLUTION IN LESS THAN 1 HOUR		++ DISSOLUTION IN 1 TO 3 HOURS		+ DISSOLUTION IN 3 TO 24 HOURS		− NO DISSOLUTION IN 24 HOURS	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
33	10	30.3	5	15.1	12	36.3	5	15.1	1	3.0

day following the inoculation, without redness of the ear, excepting at the point of inoculation. The third strain produced a typical erysipelatous lesion. On the day following the inoculation, a large part of the ear was reddened, with slight edema, the affected areas following the lymphatics. The outer portion of the ear, throughout its entire length, was delimited by a characteristic "wall," the margin being sharply marked, elevated and indurated on palpation. The temperature rose to 40.2°C., but there were no other signs of illness. After three days the ear began to heal. Other species of hemolytic streptococci produced erysipelas in rabbits with greater constancy than *S. pyogenes*.

Lancefield reported that the great majority (87.5 per cent) of her strains from human disease sources fell into her serological group A. Edwards, using Lancefield's technique, studied 26 strains from human disease and found that all of them agreed

with Lancefield's group A. From the work of these investigators, therefore, it is obvious that *S. pyogenes*, which includes 46.6 per cent of the strains of our collection from human disease sources, should logically belong to Lancefield's group A.

Every strain in our collection of *S. pyogenes* which has been grouped serologically by other investigators using Lancefield's technique, has fallen into her group A. These strains include Lancefield's scarlet fever strain C 203, Coburn's rheumatism strain RPH_1, the strain C_2b isolated from a cow associated with the Canton, Massachusetts epidemic of septic sore throat, and the strain K 158C isolated from a rabbit, all studied by Lancefield; two puerperal fever strains "Cripps" and "Watt" studied by Plummer; two strains from abscesses, ("17424" and "17318") reported by Mickle (1935); and two strains from the throats of food handlers "Jackson" and "Siniscalco," reported by Mickle. The number of strains which have been grouped according to the precipitin test is not large, but it includes several strains whose histories are suggestive of other precipitin groups. The fact that these strains agreed with *S. pyogenes* according to both phagological and serological reactions, indicates a definite correlation between the two reactions.

RELATIONSHIP OF S. PYOGENES TO THE ESTABLISHED SEROLOGICAL GROUPS

It has been shown that *S. pyogenes* is one of the species belonging to Lancefield's precipitin Group A, which contains the great majority of human pathogenic strains. When grouped according to agglutinin absorption reactions, *S. pyogenes* has been found to include a large number of serological types.

Representative strains belonging to thirteen of Griffith's (1935) serological types agreed with *S. pyogenes*. They are his types 1, 2, 4, 5, 8, 9, 11, 12, 14, 19, 24, 25 and 26. Griffith's types 15 and 18, which he found to be not clearly differentiated, each include one strain of our collection which agreed with *S. pyogenes*, and another strain which must be regarded as atypical *S. pyogenes*. The latter group will be discussed in a later paper. Griffith's type 3 is his only one in which there is a confusion of

the species when compared with the present classification. There are in our collection 5 strains which Griffith placed in his type 3. Two of them agreed with the typical *S. pyogenes*, but the remaining three belonged to three other species. Andrewes and Christie made a detailed serological study of a number of strains belonging to Griffith's types 1, 2, 3 and 4. They readily confirmed the individuality of his types 1, 2 and 4, but they could not confirm his type 3 as a distinct entity. They were of the opinion that the agglutinins which gave cross-reactions within this group were non-specific, group agglutinins. Our results confirm those of Andrewes and Christie in indicating that Griffith's type 3 is not a well defined group, but is made up of strains of various characters.

Of the serological types established by Williams and her collaborators, the strains representing the following types agreed with *S. pyogenes:* scarlet fever types I, sub I, II and IV; erysipelas types I, sub-IIa and sub-IIb.

The scarlet fever strains representing all 6 of Mueller and Klise's serological types agreed with *S. pyogenes.*

The strains from rheumatism representing Coburn and Pauli's groups I, II, III and VI agreed with *S. pyogenes.* The strains of their group V had the characters of another species. There was a discrepancy in the two classifications in regard to the two strains of their group IV, one agreeing with *S. pyogenes,* and the other agreeing with the strains of their group V.

TYPE STRAIN

Strain 1168 was chosen for the type strain, although any one of a large number of strains might have served equally well. Strain 1168 was isolated on April 27, 1935, by Dr. R. E. Otten of the Philadelphia General Hospital, from pus obtained at operation in a case of mastoiditis. The description of the type strain is as follows:

The cells are Gram-positive, and in broth culture they occur in pairs or in chains of short or medium length. On infusion agar containing 5 per cent rabbit blood, after 48 hours incubation, the colonies are smooth and discrete, the largest isolated

colonies being about 1.0 mm. in diameter. The small colonies in crowded areas are convex. The larger colonies have an opaque center and a narrow, more translucent edge, which may be somewhat flattened. A zone of clear hemolysis about 1.0 mm. wide surrounds the colonies.

Strain 1168 is sensitive to phages B/563, C/594 and C/646 in the nascent state; it is not sensitive to D/693 in the nascent state, nor to lytic filtrate B/563; final pH in glucose broth was 5.4; lactose, salicin and trehalose are fermented, mannitol and sorbitol are not fermented. There is no growth on 10 per cent bile blood agar; sodium hippurate is not hydrolyzed; the clotting of human plasma is inhibited; mice were killed in the 10^{-4} dilution in a series of rapid passages.

The type strain has been deposited in the American Type Culture Collection.

SOURCES OF HUMAN PATHOGENIC STRAINS OF S. PYOGENES

Of the 373 strains of hemolytic streptococci from human diseases in our collection, 174, or 46.6 per cent agreed with the type strain of *S. pyogenes*. The diseases with which they were associated were as follows:

Scarlet fever. Of the 120 scarlet fever strains in the collection, 74, or 61.6 per cent agreed with *S. pyogenes*. They were from the countries of three continents, as follows: From various parts of the United States, 36 strains; from Argentina, 2 strains; from Austria, 1 strain; from China, 1 strain; from England, 16 strains; from Germany, 2 strains; from Hungary, 8 strains; from Italy, 1 strain; from Russia, 5 strains; from Scotland, 2 strains.

The previous designations of those scarlet fever strains belonging to the species *S. pyogenes* which have been studied by other investigators are as follows:

The strain known as Dick I.

Williams and Gurley's strains: 2(type 1); 55(type sub-I) 114(type 2), and 28(type 4).

Mueller and Klise's strains: M 2(B I); M 9(B II); M 113(B III); M 18(B IV); M 116(B V); M 173(B V); M 10(B VI).

Griffith's strains: 130(type 1); 22(type 2); 8(type 2); Lewis (type 3);

28(type 4); 4(type 8); Blackmore (type 11); 42(type 12); J.S.5(type 15); 7¾(type 19); 13(type 26).

Mickle's (1928) strains: 60175 and 60179 from the Avon epidemic, and strains 2422, 2666, 2669 and 2670 from the Thomaston epidemic.

James' strains: S_4, S_{21}, S_{72} and S_{185}.

Smith's strains: S_3, and S_{10}.

Andrewes and Christie's strains: Moscow 41, 45, 52, 61 and 69; R. Clarke, Davy, 39, and St. Louis.

It is to be noted that the well known strains "N.Y.5," "Dick II," "Dick III" and "Dick IV," all of which are commonly used for toxin production by the manufacturers of scarlet fever antitoxin, do not agree with *S. pyogenes*. They belong to other species, which will be described in later papers of this series. It may be stated, however, that "N.Y.5" and "Dick IV" do not ferment salicin; Dick III does not ferment lactose; and Dick II and Dick III differ phagologically from *S. pyogenes*.

Erysipelas. Of the 43 erysipelas strains in the collection, 22 or 51.2 per cent agreed with *S. pyogenes*. Eighteen were isolated in various parts of the United States, 1 in Canada, and 3 in England. Among the *S. pyogenes* strains from erysipelas in our collection are Spicer, Gonshorek and Spicer's strains E/102, E/103, E/104, E/105, E/106, E/111, and E/114; and strain 769 received from the American Type Culture Collection (listed under the name *S. erysipelatis*).

Puerperal fever. Of the 62 puerperal strains in the collection, 29, or 46.8 per cent agreed with *S. pyogenes*. One strain was isolated in New York City; 5 were isolated in Canada; and the remainder were isolated in London. The previous designations of those puerperal fever strains which have been studied by other investigators are as follows:

Meleney's epidemic strain.

Griffith's strains: Franklin (type 5); and Barker (type 14).

Colebrook's strains: Collis, Mills, Ayling, Pearce, Herbert, Evans, Richards, Jackman, Patis and Timothy.

Andrewes and Christie's strains: Stevens, Jarvis, Crofts, Toms, Annie Smith, Eldridge, Unstead, Kent, Warrington, Digweed and French.

Rheumatism. Seven, or 43.7 per cent of the 16 strains from cases of rheumatism agreed with *S. pyogenes.* All 7 were strains previously studied by Coburn and Pauli. They are their strains: RC_1, RPH_1 and R_{23} (Group I); R_{22} (Group II); R_{18} (Group III); RG_3 (Group IV); R_{17} (Group VI).

Acute suppurative diseases. Nineteen, or 36.5 per cent of the 52 strains from acute suppurative diseases (meningitis, peritonitis, empyema, osteomyelitis, mastoiditis, sinusitis, otitis media, cystitis, adenitis and abscesses) agreed with *S. pyogenes.* Two strains were from London and the remainder were from Washington, D. C., Baltimore and Philadelphia.

Septic sore throat. In our collection there are strains from 21 epidemics of sore throat, with more than one strain from several of the epidemics. In an earlier paper (1935) it was reported that the majority of septic sore throat strains differ phagologically from *S. pyogenes,* but that *S. pyogenes* was involved in four of the sore throat epidemics. They were the Canton, Massachusetts epidemic of 1913; the Lee, Massachusetts epidemic of 1928, the Pittsfield, Massachusetts epidemic of 1928 (Lee and Pittsfield are only 10 miles apart), and an epidemic in a public school in England, from which Griffith obtained his strain "Symons" (type 9).

There were no human strains from the Canton epidemic available for this study, but a strain which was isolated from suspected milk agreed with *S. pyogenes.*

The 4 strains, listed as *Streptococcus pyogenes* Rosenbach in the third edition (1934) of the Catalogue of Cultures of the American Type Culture Collection were all studied, with the following results:

Strain 420 (the catalog number) agreed with the characters of *S. pyogenes* as described in this paper. Strain 4444 differed very slightly from typical *S. pyogenes.* The remaining two strains listed as *S. pyogenes* belonged definitely to other species of hemolytic streptococci, as indicated by both phagological and fermentation reactions.

STRAINS OF S. PYOGENES ISOLATED FROM COWS ASSOCIATED WITH EPIDEMICS

It is of historic interest that in 1885 Klein, who gave the specific name to the etiologic agent of scarlet fever, traced an epidemic of scarlet fever to the milk of cows on a certain farm.

Armstrong and Parran reviewed the data on milk borne epidemics in the United States to January, 1927, and found that 42 epidemics of septic sore throat and 40 epidemics of scarlet fever were on record. Twelve of the sore throat epidemics were traced to dairy farms with cows suffering with mastitis. There were no data in the records, however, which would show whether the

TABLE 2

Histories of strains of the species S. pyogenes isolated from cows

STRAIN	PREVIOUS DESIGNATION	EPIDEMIC	
		Disease	Location
567	60105	Scarlet fever	Avon, Connecticut
757	39353B		
623	27 A	Sore throat	Lee, Massachusetts
737	32 A		
773	1452	Scarlet fever	?
891	C_3b	Sore throat	Canton, Massachusetts
749	27364	Scarlet fever	Plymouth, Massachusetts

epidemics were caused by *S. pyogenes* or some other hemolytic streptococcus.

In our collection there are 7 strains from cows which agree with *S. pyogenes*. All were isolated from milk associated with epidemics, and all were previously studied by other investigators. In table 2 are given the histories of the strains. The findings of the investigators who previously studied these strains are as follows:

The Avon, Connecticut epidemic of scarlet fever (1928) was traced to a cow which, according to Mickle, showed no physical signs of udder infection at the time, but shortly afterwards began to produce milk of a gargety appearance. Mickle (1928) states that Dr. Jones of Princeton, New Jersey and Dr. Blake of New

Haven both studied the milk strains and found that they were like the scarlet fever strains. Two streptococcus cultures from the suspected milk, Nos. 567 and 757, and also a strain from the throat of a milk handler, and a strain from the throat of a patient were available for the present study, all of which were found to agree with *S. pyogenes*.

The Lee, Massachusetts epidemic of sore throat (1928) was traced to a cow which had active mastitis. Robinson and Beckler reported that they found that the strain from the cow and the strains from patients agreed with *S. epidemicus*. Williams and Gurley reported that they received strains of two serological types from the Lee epidemic. The milk strain which they studied was our No. 623. They found that it had the characteristics of scarlet fever strains. Williams and Gurley studied throat strains from the Lee epidemic and found that they agreed with *S. epidemicus*. Our results confirm the conclusions of Williams and Gurley in showing that both *S. pyogenes* and *S. epidemicus* were involved in the Lee epidemic. Two milk strains (623 and 737) and 4 throat strains of our collection agreed with *S. pyogenes*, and two throat strains agreed with *S. epidemicus*.

Strain 773 was isolated from the milk of a cow which had an injured teat and inflamed udder. A sharp outbreak of scarlet fever was traced to this cow. Jones and Little studied the milk strain extensively. They found that it was culturally indistinguishable from scarlet fever strains; that it was serologically related to scarlet fever strains, and that it produced a toxin which was neutralized by scarlet fever antitoxin. Pilot and Stocker also found that it produced a toxin neutralizable by scarlet fever antitoxin and that its toxin differed from that produced by *S. epidemicus*.

Strain 891 (C_3b) was isolated by Smith and Brown from a sample of milk from a dairy herd assumed to be the source of infection in a severe epidemic of tonsillitis in Canton, Massachusetts in 1913. Lancefield classified this strain in her group A.

Tillett studied two of the strains from cows under discussion (Nos. 773 and 891) and found that they are capable of lysing human fibrin.

The scarlet fever epidemic in Plymouth, Massachusetts, in 1929 was reported by Scamman. The ability of the Plymouth strain to produce mastitis in a cow was tested by the writer in collaboration with Dr. W. T. Miller, of the Bureau of Animal Industry, United States Department of Agriculture. The test was made five years after the isolation of the culture. Twenty-four hour infusion broth culture was injected into two quarters of the udder, in doses of 2 and 5 cc. respectively. There was no appearance of mastitis following this inoculation. Two weeks later, the other two quarters were injected with 10 and 15 cc. respectively of 24-hour broth culture. Clinical mastitis developed in the two quarters last inoculated. It gradually subsided, and 7 weeks after the last inoculation, samples of milk from the infected quarters appeared normal, and yielded only a few colonies of hemolytic streptococci. The experiment demonstrated only a mild pathogenicity of the Plymouth strain for the cow.

TABLE 3

Human carrier strains of S. pyogenes

STRAIN	PREVIOUS DESIGNATION	PREVIOUS INVESTIGATORS	REMARKS
1061	Brewer	Andrews and Christie	From throat of nurse attending puerperal case
1075	Wright	Andrewes and Christie	From throat of doctor during puerperal epidemic
1196	Jackson	Mickle	Food handler
1197	Siniscalco	Mickle	Food handler

CARRIER STRAINS

Four strains from normal human throats agreed with *S. pyogenes*. Their histories are given in table 3. Two were from food handlers without a history of contact with infected subjects. One (No. 1061) was from the throat of a nurse attending a case of puerperal fever. The patient's strain ("Toms" listed under Andrewes and Christie's puerperal strains) and the carrier strain were identical. The other carrier strain (No. 1075) was from

the throat of an attending doctor isolated during an epidemic of puerperal fever at Queen Charlotte's Maternity Hospital, London. Three strains isolated from patients were available for this study. (Strains "Warrington," "Digweed" and "French," listed under Andrewes and Christie's puerperal strains). The carrier strain and the three patients' strains were identical.

SUMMARY

According to the data presented, *Streptococcus pyogenes* Rosenbach as here defined, is the species of hemolytic streptococcus which is most commonly associated with human disease. If our collection is representative, *Streptococcus pyogenes* causes the majority of cases of scarlet fever and erysipelas, and it causes almost half of the cases of puerperal fever. It is responsible for a comparatively small percentage of epidemics of septic sore throat. The figures for each disease are given in table 4.

TABLE 4

Diseases caused by Streptococcus pyogenes

DISEASES	TOTAL NUMBER OF STRAINS FROM HUMAN DISEASE SOURCES	STRAINS OF S. PYOGENES	
		Number	Per cent
Scarlet fever	120	74	61.6
Erysipelas	43	22	51.2
Puerperal fever	62	29	46.8
Rheumatism	16	7	43.7
Acute suppurative diseases	52	19	36.5
Epidemic sore throat	24	4	16.6
Other miscellaneous diseases	56	19	33.9
Total	373	174	46.6

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THE UTILIZATION OF LACTOSE BY ESCHERICHIA COLI-MUTABILE

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Atypical strains of colon bacilli which do not exhibit characteristic fermentation of lactose have long been recognized. Recently, Dulaney and Michelson (1935) and Michelson and Dulaney (1936) have described strains of *Escherichia coli-mutabile* which were isolated from the feces of infants suffering from diarrhea. They were able to demonstrate in lactose broth cultures of these organisms that acid production, as indicated by brom-thymol-blue indicator, was coincident with the appearance of red[1] variants of the initial white form of the organism. This was determined by periodic inoculation of Endo's agar with the broth cultures, the distribution of white and red colonies on the plates being taken as representing the approximate ratio of white to red organisms in the culture at time of transfer.

The present study was undertaken to determine whether lactose is utilized in cultures of *Escherichia coli-mutabile* only after red variants have been produced, and to correlate quantitatively lactose utilization with the appearance of red forms. Lewis (1934) remarks: "Much has been written on the problem of whether the specific sugar or alcohol is attacked from the beginning of a culture or only after a period of contact with it but no very definite experimental evidence is available and opinions differ."

Lewis found that eight out of nine strains of colon bacteria

[1] The terms "red" and "white" are used in the present paper to define, respectively, the lactose fermenting and non-lactose fermenting forms of *E. coli-mutabile*, following the terminology of Dulaney and Michelson (1935).

variable to lactose were able to grow in a synthetic medium containing lactose as the sole source of carbon. With one strain of *Aerobacter cloacae-mutabile* the maximum number of organisms was reached after an incubation period of 60 hours although the medium did not become acid until after 84 hours. These observations appear to demonstrate that these organisms can use lactose for growth and energy at a time when they are not producing acid by fermentation of lactose.

Jones, Orcutt, and Little (1932), working with several strains of atypical colon bacilli, concluded from quantitative lactose determinations that lactose was utilized from the beginning of culture, even though the lactose broth remained alkaline for periods varying from 3 or 4 days to 2 or 3 weeks. The failure of the medium to become acid with the rapidity characteristic of *E. coli* was attributed to the production of alkali (ammonia?) which neutralized the acids assumed to have been produced by fermentation. Upon rapid passage in lactose broth certain of the strains gave rise to organisms which readily fermented lactose. The slow lactose fermenting organisms differed, however, from *E. coli-mutabile* in that papillae or buds containing organisms capable of rapidly producing acidity in lactose broth were not formed when the organisms were grown on lactose agar plates.

The failure of *E. coli-mutabile* to produce acidity within 24 hours when grown in lactose broth is conventional evidence of failure of the organisms to attack lactose during this period. The results of the authors cited above however, suggest that certain colon organisms atypical in their behavior toward lactose are able to metabolize lactose and that the type of metabolism is not the usual acid fermentation characteristic of colon organisms. Merrill (1930), working with members of the Genus *Mycobacterium*, pointed out the fallacy in concluding that carbohydrates are not utilized simply because the medium fails to become acid. It is conceivable then, though doubtful, as the present paper will show, that both the red and white strains of *E. coli-mutabile* are able to utilize lactose and that the difference in their behavior is due to a difference in type of sugar metabolism.

DETERMINATION OF SUGAR IN BACTERIOLOGICAL MEDIA

We have employed the official method of the Association of Official Agricultural Chemists (1925) for lactose determination except that the Cu_2O is ignited to CuO before being weighed. The lactose equivalent of the CuO formed is determined by use of Munson and Walker's table. All lactose values given in this paper are reported as anhydrous lactose.

TABLE 1

Determination of lactose in lactose broth

SAMPLE NUMBER	LACTOSE CONCENTRATION	LACTOSE IN 50 CC. SAMPLE (CALCULATED)	LACTOSE (FOUND)	RECOVERY
	per cent	*mgm.*	*mgm.*	*per cent*
1	0.250	125	127.9	102.3
	0.250	125	127.2	101.8
2	0.200	100	101.6	101.6
	0.200	100	101.6	101.6
3	0.150	75	75.5	100.7
	0.150	75	75.5	100.7
4	0.100	50	50.1	100.2
	0.100	50	50.1	100.2
5	0.050	25	20.7	82.8
	0.050	25	18.8	75.2
6	0.025	12.5	1.3	
	0.025	12.5	1.9	
7	0	0	2.8	
	0	0	0.6	

The accuracy of this method when applied to the determination of lactose in broth is shown in table 1. Broth containing 0.4 per cent Difco dehydrated nutrient broth and 0.4 per cent sodium chloride was mixed in equal volumes with lactose solutions of varying concentrations. The resulting solutions were subjected to analysis for lactose by the method mentioned above. The final concentration of broth in the solution is 0.2 per cent, the equivalent of a 1:4 dilution of the usual 0.8 per cent nutrient

broth. In some of our sugar determinations in 1 per cent lactose broth we have used a 1:3 dilution and in others a 1:6 dilution. The higher dilution is preferred because of the smaller bulk of Cu_2O formed.

Our results show that lactose can be determined with fair accuracy in the presence of broth. Determinations on broth to which no sugar was added give a small blank. The lactose values reported in this paper have not been corrected for the slight reducing power of the broth. Accordingly, the values are a little high in all cases in which the lactose concentration is greater than 1 mgm. per cubic centimeter. Below this concentration the results are inconsistent. The upper limit of lactose concentration detectable by this method was not determined though the table of Munson and Walker indicates that as much as 6 mgm. per cubic centimeter may be used.

STUDIES ON LACTOSE UTILIZATION BY E. COLI-MUTABILE

In order to determine whether the white form of *E. coli-mutabile* utilizes lactose from the beginning of growth or only after the appearance of red or lactose-fermenting organisms, the following experiment was designed. Difco dehydrated nutrient broth containing 0.8 per cent NaCl was prepared and the pH adjusted to 7.0–7.2. Three hundred and fifty cc. amounts of broth were placed in 500 cc. Erlenmeyer flasks and sufficient 10 per cent lactose solution, sterilized separately, added to give a concentration of 1 per cent. The media were inoculated with saline suspensions of Garrett red and Garrett white colonies from Endo's plates and incubated at 37°C. At intervals samples were withdrawn for quantitative lactose determination and for inoculation of Endo's plates to determine the time of appearance of lactose-fermenting variants in the culture of the white strain. The percentage of white and red colonies was determined at each interval. The results, presented in table 2, demonstrate that the white form of *E. coli-mutabile* utilizes very small quantities of lactose, if any, before lactose fermenting variants can be demonstrated. The red strain uses lactose from the start.

It has been observed that the amount of lactose eventually used

by the white strain slightly exceeds the amount used by the corresponding red strain. If, as our results indicate, the white strain must derive its energy from the nitrogenous components of the medium the amount of ammonia formed in a culture of the white strain might be expected (Kendall, 1923) to exceed the amount formed by the red strain. This increased amount of ammonia should then allow more sugar to be used before the limiting pH is attained.

These possibilities were examined in the following experiment. Three hundred and fifty cubic centimeters of lactose broth prepared as above were placed in 500 cc. Erlenmeyer flasks. Garrett red, Garrett White, Rimmer white, and Terrell white were the

TABLE 2

Correlation of lactose utilization by E. coli-mutabile with its dissociation to lactose fermenting variants (reds)

TIME OF INCUBATION	LACTOSE CHANGES		PER CENT OF REDS PRESENT IN THE CULTURE OF GARRETT WHITE
	Garrett red	Garrett white	
hours	*mgm. per 100 cc.*	*mgm. per 100 cc.*	
7.5	−24	+ 8	0
19	−197	−12	0
24	−246	−12	0
33	−277	−45	12
41	−295	−233	50
48	−295	−314	75

organisms used here. The inocula were 0.2 cc. of 7-hour broth cultures of whole colonies obtained from Endo's plates. The temperature of incubation was 37°C. At stated intervals samples were withdrawn and used for the following determinations: pH, lactose, ammonia N, amino-N, and the inoculation of Endo's plates. The pH determinations were made with the quinhydrone electrode.

Ammonia and amino-nitrogen determinations. Fifteen cubic centimeters of the medium were added to 1 cc. of N HCl and allowed to stand until the determinations could be performed. The pH, 1.4 to 1.5, of this mixture serves to prevent further bacterial growth. The amino-N determinations were performed

TABLE 3

Certain metabolic activities of white and red strains of E. coli-mutabile

(Lactose, ammonia-N, and amino-N are reported in milligrams per 100 cc.)

INTERVAL	GARRETT RED					GARRETT WHITE				
	pH	Lactose	Amino-N	Ammonia-N	Plates	pH	Lactose	Amino-N	Ammonia-N	Plates
hours										
0	7.0	1,022	20	1.2	All red	7.0	1,030	20	1.4	All white
8	6.5	889	19	2.2	All red	6.9	1,022	21	2.4	All white
16	5.7	815	19	2.3	All red	6.7	1,007	22	3.3	All white
24	4.6	791	18	2.2	All red	7.0	1,022	22	4.9	All white
32	4.5	778	18	2.4	All red	7.3	1,030	22	8.4	2% red
40	4.4	799	19	2.4	All red	5.7	918	21	7.4	50% red
48	4.4	778	19	2.4	All red	4.9	822	21	7.7	50% red
72	4.4		20	2.4	All red	4.6	769	19	7.7	40% red
	RIMMER WHITE					TERRELL WHITE				
0	7.0	1,022	21	1.2	All white	7.0	1,022	20	1.3	All white
8	7.2	1,007	19	2.3	All white	7.3	1,000	20	2.4	All white
16	6.8	1,000	22	2.7	All white	6.9	1,000	20	3.3	All white
24	6.7	1,000	20	4.6	10% red	6.8	992	20	5.3	6% red
32	6.3	874	19	6.6	50% red	5.9	858	19	6.9	60% red
40	4.8	799	20	6.9	65% red	4.7	785	21	7.0	77% red
48	4.7	791	21	7.0	60% red	4.6	762	19	7.1	73% red
72	4.7	785	19	7.0	98% red	4.5	747	20	7.1	75% red

TABLE 4

Comparison of pH and ammonia production by Garrett white and Garrett red when grown in plain broth

TIME OF INCUBATION	GARRETT WHITE		GARRETT RED		CONTROL (UNINOCULATED)	
	Ammonia N	pH	Ammonia N	pH	Ammonia N	pH
hours	*mgm. per 100 cc.*		*mgm. per 100 cc.*		*mgm. per 100 cc.*	
0	1.4	7.1	1.4	7.1	1.4	7.1
8	2.7	6.7	2.8	6.7		7.1
16	3.9	6.7	3.9	6.7		7.1
24	6.9	7.1	8.2	7.2	1.4	7.1
32	9.2	7.5	9.7	7.5		7.1
40	9.1	7.7	9.6	7.7		7.1
48	9.1	7.8	9.5	7.8	1.4	7.1
72	9.6	8.1	10.2	8.0	1.4	7.1
96	12.9	8.5	14.0	8.5	1.5	7.1

on aliquots of this mixture using the Sörenson titration as described by Northrop (1926). The ammonia determinations were performed on aliquots by nesslerization after separation of the ammonia by aeration (Williams and Nash, 1933).

The results of this experiment are presented in table 3. It is again demonstrated that lactose utilization is delayed until the appearance of lactose-fermenting variants. Ammonia production in cultures of the white strains is much greater than in the culture of the red strain. This increased ammonia production is reflected in the pH changes observed. The amino-N values show little change.

Additional evidence that the white strain, before the appearance of red variants, acts in lactose broth as in plain broth is given by the results in table 4. Garrett white and Garrett red were inoculated into broth identical with that used above except for the omission of lactose. Ammonia and pH determinations were performed at intervals as in the preceding experiment. The results demonstrate the similar behavior of Garrett white and Garrett red as regards pH changes and ammonia production when grown in plain broth. A comparison of the pH and ammonia values of Garrett white when grown in lactose broth (table 3) and when grown in plain broth (table 4) demonstrates the similarity of behavior for the first 32 hours, e.g., until the appearance in the lactose broth of lactose-fermenting variants.

DISCUSSION

Our results shed little light on the rôle of lactose in stimulating variation in *E. coli-mutabile*. It is shown adequately that the white form of *E. coli-mutabile* does not attack lactose with the rapidity characteristic of the red organisms. These findings are not strictly comparable with the results of Jones, Orcutt, and Little. Their lactose determinations were performed only after an incubation of 2 days. At this period their atypical (slow) lactose fermenting organisms had utilized appreciable quantities of lactose. With the strains of *E. coli-mutabile* used in our studies the difference in lactose utilization by the white and red forms is striking during the first day. By the end of the second day the

culture originally containing the white form may have used as much lactose as the red form. We have shown that lactose utilization and acid production by *E. coli-mutabile* when grown in lactose broth are closely correlated with the appearance of lactose fermenting variants. Additional evidence that the white strains utilize nitrogenous compounds rather than lactose as their source of energy is afforded by the similarity in ammonia production and pH changes whether the organism is grown in plain broth or lactose broth. We believe the delayed appearance of acid and gas when *E. coli-mutabile* is grown in lactose broth is due principally to delayed lactose fermentation rather than to production of alkali. The white strain does, however, produce more ammonia in lactose broth than the red strain. We take this fact as additional evidence of delayed fermentation of lactose. Our results do not eliminate the possibility that traces of lactose are utilized by *E. coli-mutabile*. Methods for lactose determination are not sufficiently accurate to determine whether traces are being used when the lactose concentration is 1 per cent. Lewis' method of growing *E. coli-mutabile* and other mutabile organisms in a synthetic medium containing lactose as the sole source of carbon is better for determining the availability of lactose to the organisms. It may be pointed out, however, that of the nine strains tested by Lewis one failed to grow in synthetic medium and this was the only fecal strain of *E. coli-mutabile in his series*. All of the strains used in our studies were of fecal origin.

SUMMARY

1. The official gravimetric method for determining lactose of the Association of Official Agricultural Chemists has been shown to give accurate lactose determinations in lactose broth without any preliminary treatment to remove nitrogenous substances.

2. The white form of *Escherichia coli-mutabile* when grown in lactose broth utilizes very small quantities of lactose, if any, before lactose-fermenting variants can be demonstrated. Appreciable lactose utilization and production of acid and gas by cultures of *Escherichia coli-mutabile* in lactose broth are so closely

correlated with the appearance of lactose-fermenting variants that we are forced to conclude that the fermentative changes are due to these variants.

3. The white and red forms of *Escherchia coli-mutabile* when grown in plain broth produce like changes in the ammonia content and pH of the medium. The ammonia production and pH changes produced by the white form are similar whether the organism is grown in plain or lactose broth. When grown in lactose broth the white strain produces much more ammonia than the red strain. We interpret these findings as additional evidence that the white form of *Escherichia coli-mutabile* lacks a capacity to utilize lactose and that, consequently, the organism obtains its energy from the nitrogenous fractions of the medium.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

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STUDIES OF THE STREPTOCOCCUS ZYMOGENES. *Ruth Alida Thomas*, Department of Applied Pathology and Bacteriology, Cornell University Medical College and New York Hospital.

Streptococcus zymogenes has received little attention from bacteriologists but it is encountered not infrequently in the blood of patients suffering from a subacute form of endocarditis, indistinguishable either clinically or pathologically from that produced by *Streptococcus viridans*. It is an oval, Gram-positive coccus which tends to occur in pairs or short chains and may be identified by reason of its marked enzymatic activity. The simplest differential media are gelatin and litmus milk. Gelatin is liquified readily and, in litmus milk, the organism grows characteristically with prompt decolorization of the litmus, coagulation of the casein, and liquefaction of the clot thus formed. The enzymes may be demonstrated in broth culture filtrates. A casein medium is also suggested to facilitate isolation from mixed cultures. All of our strains produce a definite greenish coloration of red blood cells and no hemolysis. In this respect they differ from the hemolytic strains which have been reported by Frobisher and Denny and Torrey and Montu as *Streptococcus zymogenes*. Four hemolytic strains obtained from Dr. Torrey and the American Type Museum proved indistinguishable culturally or biochemically from our strain except in their action on blood. Serologically they are apparently closely related. Experiments are now underway in which we hope to show the factors influencing this apparent variation.

HYPERSENSITIVITY AND IMMUNITY IN STREPTOCOCCUS INFECTION. *Murray Angevine*, Department of Pathology, Cornell University Medical College.

Rabbits were given repeated intradermal injections of living avirulent hemolytic streptococci (strain B_1) and later tested for hypersensitiveness with an homologous streptococcus filtrate. The same organism was then injected into the skin of normal and hypersensitive rabbits. In the normal animals streptococci multiplied for a short time and disappeared from the site of injection in twelve hours. They reached the adjacent lymph nodes in moderate numbers and were occasionally recovered from the blood and spleen. In the hypersensitive animals there was more multiplication at the site of the inoculation and organisms were recovered over a longer period, whereas relatively few reached the adjacent lymph node and none were recovered from the blood stream or spleen.

Similar experiments were performed

with a highly virulent (strain "H") streptococcus. With repeated injections the skin lesions became smaller and the rabbits reacted moderately to test doses of filtrate. Virulent organisms in normal rabbits produced extensive abscesses from which large numbers of streptococci were recovered over a period of 21 days; many reached the lymph nodes, blood and spleen, and an occasional animal died with a bacteremia. When inoculated into previously injected rabbits, there was little multiplication and no bacteria were recovered from the skin after 48 hours. They reached the adjacent lymph node in small numbers and none were recovered from the blood or spleen. Histological sections confirm the bacteriological findings with both strains of streptococci used.

MOTION PICTURE OF THE DEVELOPMENT OF BACTERIA FROM A SINGLE CELL. *Morton C. Kahn, Edward K. Taylor and Helen Schwarzkopf*, Department of Public Health and Preventive Medicine, Cornell University Medical College, New York City.

The motion pictures reveal the fact that three bacteria, isolated singly and all the progeny of one cell, multiply by three distinct types of growth processes. The microörganism from an "S" colony in this instance, multiplies by rapid segmentation into extremely minute bodies, some of which later elongate into much larger organisms, all of this taking place in the microdroplet which contains the original single bacterium.

A single microörganism from an "R" colony, grew out into a filament some 95 micra in length before eventually splitting into a number of rods.

A single microörganism isolated from a second "R" colony multiplied by direct fission in the usual sense of the term. These films serve to illustrate some of the complexities of the problem of bacterial growth as the three types of growth here illustrated are from single organisms all derived from the same parent cell. As concerns the growth of the single cell isolated from the "S" colony, it will be found that it resembles in certain respects the cycle described by one of us (Kahn) for the tubercle bacillus. It serves to prove that forms much smaller than usual are not necessarily evolved by crowding of the environment, and secondly that some of these small bacterial coccoid forms or granules are capable of elongation without necessarily being replanted on fresh medium.

THE REACTION OF CRYSTAL VIOLET WITH STAPHYLOCOCCI. *George H. Chapman*, Clinical Research Laboratory, 604 Fifth Avenue, New York, N. Y.

Staphylococci grew poorly on proteose lactose agar containing 1:300,000 crystal violet (violet agar). A study of the violet agar reaction revealed that only tetra-, penta- and hexamethyl pararosanilines give the color reaction with staphylococci. The higher homologs seemed to give best results. The reaction proved to be so specific that improperly labeled dyes containing any of these three compounds could be detected by this method.

To determine whether the violet agar reaction was caused by the action of polysaccharides, pure samples were tested with leuco compounds. Preliminary studies indicate that Type A and Type B polysaccharides act differently. While both are precipitated by the leuco bases of malachite green, brilliant green and crystal violet, the latter precipitate redissolves, giving rise to blue or purple

compounds with different solubilities. These results suggest an oxidation-reduction mechanism.

ELECTIVE LOCALIZATION IN RABBITS OF CULTURES FROM PATIENTS WITH CERTAIN INFLAMMATORY EYE DISEASES (PRELIMINARY REPORT). *Conrad Berens, Edith L. Nilson and George H. Chapman*, Lighthouse Eye Clinic of the New York Association for the Blind and the Clinical Research Laboratory, New York, N. Y.

Attempts were made to demonstrate elective localization in rabbits by cultures from the nose, throat and other foci of patients with certain inflammatory ocular diseases. Cultures from patients free from eye disease were used as controls.

In addition to the original whole cultures in glucose brain broth, the individual organisms were isolated and pure cultures of the different types of bacteria were injected into other rabbits. The usual procedure was to inject 5.0 cc. of culture intravenously. Rabbits were observed closely up to 48 hours and, if no effect was noted, they were discarded after eight days. Iritis usually appeared within 12 to 24 hours. Conjunctivitis (usually from toxemia) was ignored. The effective dose often caused death of the animals within a few days. In almost all cases in which iritis was produced by the original culture, when organisms were isolated from the original culture, some of them also localized in the eyes. In addition to streptococci, iritis was also produced by pure cultures of enterococci, *Escherichia*, *Aerobacter*, staphylococci and a non-lactose fermenter.